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ÉLABORATION DE NOUVEAUX BIOMATÉRIAUX ANTIBACTÉRIENS À BASE DE CHITOSANE PAR LE PROCÉDÉ D'ÉLECTROFILAGE

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THÈSE PRÉSENTÉE EN VUE DE L'OBTENTION

DU DIPLÔME DE PHILOSOPHIAE DOCTOR

(GÉNIE CHIMIQUE)

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UNIVERSITÉ DE MONTRÉAL

ÉCOLE POLYTECHNIQUE DE MONTRÉAL

Cette thèse intitulée:

ÉLABORATION DE NOUVEAUX BIOMATÉRIAUX ANTIBACTÉRIENS À BASE DE CHITOSANE PAR LE PROCÉDÉ D'ÉLECTROFILAGE

présentée par : ARKOUN Mounia

en vue de l'obtention du diplôme de : Philosophiae Doctor

a été dûment acceptée par le jury d'examen constitué de :

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DÉDICACE

À mes précieux parents

À mon tendre mari

À notre douce petite fille

À mes très chères sœurs

À mes adorables nièces et neveux.

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Lætab ur yettnahsab اللهم انفعـــنى بمـا علمتـــنى و علمـنى ما ينفعـــــنى « وَقُل رَّبِّ زِدْنى علْمًا »

RÉSUMÉ

Actuellement, des efforts considérables sont entrepris par les chercheurs afin de concevoir des matériaux d'emballages actifs, basés sur des technologies propres et respectueuses de l'environnement. L'objectif étant de satisfaire la demande des consommateurs pour des produits alimentaires sûrs, sains, pas ou peu transformés et sans conservateurs, avec la perspective de réduire le gaspillage et les intoxications alimentaire, essentiellement causés par le développement d'une flore d'altération et pathogène dans les aliments.

Le chitosane est un polymère d'origine naturelle possédant de puissantes propriétés antimicrobiennes contre un large spectre de bactéries, levures et moisissures. L'inconvénient principal du chitosane est sa mauvaise aptitude à la mise en forme par les procédés industriels typiques des polymères. En effet, le chitosane étant un biopolymère thermosensible, et les procédés de mise en forme requérant des températures élevées, celui-ci se dégrade bien avant de fondre.

Cette thèse vise donc à élaborer des matériaux antibactériens à base de chitosane par le procédé d'électrofilage. L'électrofilage du chitosane dans le but de préparer des nanofibres est un processus prometteur qui a suscité beaucoup d'intérêt durant les dernières années. Hormis leur biocompatibilité et leur activité bactéricide, les nanofibres de chitosane (CNFs) ainsi obtenues possèdent des propriétés très recherchées parmi lesquelles un faible diamètre (40 nm, proche de celui des fibres de collagène), une grande surface spécifique (jusqu'à 500 m2/g) et une importante porosité (~ 80 %).

Le présent travail s'articule autour de trois grandes étapes. Étant donné que, pour être actives, les CNFs doivent être en contact direct avec l'aliment emballé, comprendre leur mécanisme d'action est alors crucial dans la lutte contre le gaspillage et les intoxications alimentaires. La première étape consiste donc à préparer les CNFs et à examiner leur mécanisme d'action antimicrobien in vitro, sur des souches bactériennes commensales et pathogènes les plus souvent incriminées dans l'altération microbiologique des aliments. L'activité bactéricide des CNFs a été investiguée contre deux bactéries à Gram négatif, *Escherichia coli* et *Salmonella enterica* serovar Typhimurium et deux bactéries à Gram positif, à savoir *Staphylococcus aureus* et *Listeria innocua*. La sensibilité/résistance des souches étudiées a été évaluée en termes de type de Gram, de l'hydrophobicité et de la densité de charge cellulaires de surface, mais aussi en fonction de la

pathogénicité. Les résultats indiquent que l'activité antibactérienne des CNFs dépend de la protonation des groupements amines, et ce indépendamment du type bactérien. Le mode d'action des CNFs s'est révélé être bactéricide et non bactériostatique. Quant à la sensibilité des souches, celle-ci s'est avérée indépendante du type bactérien, tandis que l'hydrophobicité et la densité de charge de surface n'expliquent qu'en partie pour quoi certaines souches sont plus sensibles que d'autres à l'action des CNFs. L'ordre de sensibilité des souches était comme suit: *E. coli* > *L. innocua* > *S. aureus* > *S.* Typhimurium, les souches non pathogènes ou les moins virulentes étant les plus sensibles à l'action des CNFs.

La deuxième étape étudie l'effet des CNFs sur l'intégrité de la membrane bactérienne. Les résultats montrent clairement que les CNFs, chargées positivement interagissent avec la paroi bactérienne, de charge opposée, induisant ainsi une déstabilisation de l'homéostasie de la cellule. Le mécanisme d'action des CNFs implique une perméabilisation de la membrane cellulaire. Le relargage du contenu cytosolique, incluant protéines, enzymes et ADN dans le milieu extracellulaire est une indication de la perméabilisation et perforation de la membrane plasmique, puisque la formation de pores a été observée en microscopie électronique à transmission.

Dans la troisième et dernière étape, la préparation de matériaux d'emballage à base nanofibres de chitosane en vue d'une application concrète est investiguée. Pour ce faire, les solutions à base de chitosane ont été électrofilées sur la face interne d'un emballage conventionnel, en contact direct avec l'aliment à emballer. L'effet de la teneur en solvant et de l'ajout de faibles proportions d'oxyde de polyéthylène (PEO) sur l'élasticité, la densité de charge et la structure conformationnelle des chaînes de chitosane et son électrofilabilité ont d'abord été étudiés. Par la suite, l'efficacité bactéricide des emballages activés ainsi obtenus a été évaluée in vitro et dans des conditions réelles sur de la viande fraîche. Les résultats montrent qu'une bonne élasticité, atteinte à une teneur optimale en acide acétique de 50 % (v/v) et l'ajout de PEO à faible proportion (10-20 % p/p) facilitent la formation des nanofibres. Les résultats des tests in situ démontrent le potentiel prometteur (R = 95 %) des emballages à base de CNFs dans la lutte contre le gaspillage et les intoxications alimentaires d'origine bactériennes. De plus, l'allongement (d'une semaine) de la durée de vie de la viande fraîche est une propriété très recherchée dans l'industrie alimentaire et que les CNFs ont démontrée avec succès.

ABSTRACT

Currently, considerable efforts are being undertaken by researchers to design active packaging materials on the basis of greener, cleaner and ecofriendly technologies. The goal is to satisfy consumers' demand for safe, healthy, unprocessed or minimally processed and preservative free food products, with the prospect of reducing food waste and poisoning, mainly caused by the development of alteration and pathogenic flora in food.

Chitosan is a natural polymer exhibiting strong antimicrobial properties against a broad spectrum of bacteria, yeasts and molds. The main disadvantage of chitosan is its poor processability in typical industrial processes for polymers. Indeed, being a thermosensitive biopolymer while polymer processing requires high temperatures, chitosan thermally degrades before it melts.

This thesis aims at developing chitosan-based antibacterial materials by the electrospinning process. Electrospinning of chitosan for the purpose of preparing nanofibers is a promising method that has attracted much interest in recent years. Besides their biocompatibility and bactericidal activity, the electrospun chitosan nanofibers (CNFs) exhibit highly desirable properties including a small diameter (40 nm, close to that of collagen fibers), a high surface area (up to 500 m2/g) and a high porosity (~ 80 %).

The present work has focused on three main steps. Since in order to be active, CNFs must be in direct contact with the packaged food, understanding their mechanism of action is therefore crucial in the fight against food waste and poisoning. The first step was to prepare CNFs and examine their antimicrobial mechanism of action in vitro against commensal and pathogenic bacterial strains most often incriminated in microbiological alteration of food. The bactericidal activity of CNFs was investigated against two Gram-negative bacteria namely *Escherichia coli*, *Salmonella enterica* serovar Typhimurium and two Gram-positive bacteria, *Staphylococcus aureus* and *Listeria innocua*. The susceptibility/resistance of the strains was evaluated in terms of Gram-type, cell surface hydrophobicity and charge density, as well as pathogenicity. The results indicated that the antibacterial activity of CNFs depends on the protonation of the amine groups, independently of bacterial type. The mode of action of CNFs revealed to be bactericidal and not bacteriostatic. Besides, the susceptibility of the strains was found to be independent of the Gram-type, while the hydrophobicity and the surface charge density explained only partly why some strains were more susceptible to the action of CNFs. The order of strain susceptibility was as

follows: *E. coli* > *L. innocua* > *S. aureus* > *S.* Typhimurium, the non-pathogenic or least virulent strains being the most susceptible to the action of CNFs.

The second step examines the effect of CNFs on the bacterial membrane integrity. The results clearly showed that the positively charged CNFs interact with the oppositely charged bacterial cell wall, thus inducing destabilization of the homeostasis of the cell. The mechanism of action of CNFs also involves permeabilization of the cell membrane. Moreover, the release of cytosolic compounds including proteins, enzymes and DNA in the extracellular medium, is an indication of the permeabilization and perforation of the plasma membrane, since pore formation was observed in transmission electron microscopy (TEM).

In the third and last step, the preparation of CNF-based packaging (CNFP) materials as a concrete application was investigated. To do so, chitosan-based solutions were electrospun on top of the internal face of a conventional packaging, in direct contact with food. The effect of solvent strength as well as the addition of poly(ethylene oxide) (PEO) on the elasticity, charge density and conformational structure of chitosan chains and its electrospinnability was firstly studied. Thereafter, the bactericidal efficiency of the activated packaging was evaluated in vitro and under real conditions with fresh meat. The results show that a good elasticity, achieved at an optimum acetic acid content of 50 % (v/v) and by the addition of low proportions of PEO (10-20 wt %) facilitated the fiber formation process. The results of the in situ tests demonstrate the promising potential (R = 95%) of the CNFP in the fight against food waste and poisoning caused by bacterial contamination. In addition, the extension of the shelf-life (one week) of the tested fresh meat is a very sought-after property in the food industry and which CNFs have successfully demonstrated.

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AB	Antibactérien
AcOH	Acide acétique
ADN	Acide désoxyribonucléique
ARN	Acide ribonucléique
Amp	Ampicilline
ASTM	American Standards for Testing and Materials
BATH	Bacterial Adhesion To Hydrocarbon (adhésion bactérienne sur hydrocarbure)
BHI	Bouillon cœur-cerveau (Brain Heart Infusion)
bp	Paires de bases (base pair)
C_e	Concentration d'enchevêtrement
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
CMB	Concentration minimale bactéricide
CMI	Concentration minimale inhibitrice
CS	Chitosane
CS/PEO	Mélanges de Chitosane et PEO
Ctrl	Contrôle (négatif ou positif)
DA	Degré d'acétylation
Da	Dalton
DDA	Degré de déacétylation
DO_{600}	Densité optique à 600 nm
EtOH	Éthanol
HCl	Acide hydrochlorique

IZD	Diamètre de la zone d'inhibition
Kan	Kanamycine
kDa	Kilo Dalton
Lac*Z*	Gène codant pour l'activité enzymatique β-galactosidase
LB	Luria-Bertani
LPS	Lipopolysaccharide
LTA	Acide lipoteichoïque
MEB	Microscopie électronique à balayage
MET	Microscopie électronique à transmission
MHA	Muller Hinton agar
MilliQ	Eau ultra pure
M_n	Poids moléculaire moyen en nombre
M_v	Poids moléculaire moyen en viscosité
M_w	Poids moléculaire moyen en poids
Na_2CO_3	Carbonate de sodium
NaAc	Acétate de sodium
NaCl	Chlorure de sodium
NaOH	Hydroxyde de sodium
CNFs	Nanofibres de chitosane
ONPG	*O*-nitrophenyl-β-galactoside
PBS	Tampon phosphate salin
PEO	Oxyde de polyéthylène
PM	Poids moléculaire
R (%)	Taux de réduction

SCD	Densité de charge de surface
SDS	Sodium dodécyl sulphate
SDS-PAGE	Électrophorèse sur gel de polyacrylamide en présence de sodium dodécyl sulfate
β-gal	β-galactosidase
UFC	Unités Formant Colonies

CHAPITRE 1 INTRODUCTION

Notre société est de plus en plus consciente des problèmes écologiques et climatiques que nos activités industrielles engendrent sur l'environnement. Il est donc primordial de trouver des alternatives et des solutions de rechange pour limiter au mieux le réchauffement climatique, réduire notre empreinte carbone et diminuer les émissions de gaz à effet de serre (GES), pour une croissance économique verte et un développement durable.

Une partie de la solution consiste à promouvoir le développement et l'utilisation de ressources renouvelables, provenant idéalement de la récupération de sous-produits et de résidus issus de l'alimentation, de l'agriculture, de la pêche et/ou de la biomasse, entre autres. Récemment, un intérêt considérable s'est porté sur le développement et la valorisation de polymères naturels et biosourcés comprenant des polysaccharides tels que la cellulose, l'amidon, la chitine et le chitosane, la pectine, l'alginate, l'agar-agar, les carraghénanes, la gomme Arabique, le xanthane mais aussi des protéines et des lipides et bien d'autres [1]. Ces matériaux offrent des propriétés diversifiées dans la mesure où ils sont biodégradables, biocompatibles, parfois comestibles et surtout non toxiques.

Par ailleurs, l'altération de la qualité microbiologique des aliments, de la production/transformation jusqu'à la consommation, en passant par la distribution est un enjeu de taille auquel l'industrie alimentaire fait face constamment. Le plus souvent causée par le développement d'une flore d'altération, l'expiration de la date limite de conservation (DLC) est à l'origine d'un gaspillage alimentaire alarmant. En outre, l'éclosion de bactéries pathogènes cause des toxi-infections alimentaires parfois très sévères en cas d'ingestion des aliments contaminés, sans parler du fait que cela peut nuire à l'image et à la réputation de la compagnie impliquée. Par conséquent, prolonger la DLC des aliments emballés, tout en améliorant leur sécurité sanitaire est crucial, à la fois pour l'industrie alimentaire et les consommateurs.

Une solution commune à ces deux fléaux majeurs, que sont le gaspillage et les intoxications alimentaires serait l'emballage antibactérien. En effet, la fonction ultime d'un emballage antibactérien, doté d'une activité bactériostatique/bactéricide est d'inhiber/tuer les microorganismes responsables de l'insalubrité de l'aliment emballé.

En plus d'être biodégradables, le chitosane et ses dérivés présentent un large spectre d'activité antimicrobienne. Cette propriété offre un potentiel intéressant pour l'utilisation de matériaux à base de chitosane comme agents antimicrobiens pour le revêtement de différentes surfaces afin de prévenir et/ou éliminer les infections microbiennes. Lorsqu'il est solubilisé dans des solvants faiblement acides, le chitosane possède une densité de charges positive importante due à la protonation de ses groupements amines. Cette propriété lui confère un grand nombre de fonctionnalités fort attrayantes en vue d'une application dans divers domaines. Une des fonctionnalités les plus exploitées du chitosane est son activité antimicrobienne contre une large gamme de bactéries, levures et moisissures. Cette propriété fait du chitosane un candidat idéal dans le domaine biomédical ou encore l'emballage alimentaire.

L'inconvénient principal du chitosane est sa mauvaise aptitude à la mise en forme. Selon Matet *et al*. [2] le chitosane montre une température de dégradation inférieure à son point de fusion, ce qui empêche la production de films de chitosane à grande échelle par extrusion et leur développement dans plusieurs applications. En outre, les applications potentielles des solutions et films de chitosane obtenus par évaporation de solvant sont limitées en raison des propriétés mécaniques et barrière médiocres.

L'électrofilage du chitosane dans le but de préparer des nanofibres est un processus prometteur qui a suscité beaucoup d'intérêt durant les dernières années [2-9]. Le faible diamètre des CNFs (40 nm, semblable aux fibres de collagène), leur surface spécifique impressionnante (10-500 m^2/g), leur importante porosité (~ 80 %), leur biocompatibilité et leurs propriétés fonctionnelles les rendent particulièrement attrayantes pour diverses applications parmi lesquelles le génie tissulaire [10], les pansements [11], la libération contrôlée de médicaments [10], la thérapie génique [12], la filtration de l'eau [13], l'immobilisation d'enzymes [14], les biosenseurs dans le cadre du diagnostic [10], et bien d'autres.

Bien que les propriétés antimicrobiennes des solutions de chitosane aient été largement rapportées et que plusieurs études s'y soient penchées, avec une grande majorité portant sur des solutions et films à base de chitosane [15-21], l'activité antibactérienne des CNFs a reçu beaucoup moins d'attention et n'a été étudiée que superficiellement. De plus, si seules quelques études ont investigué le mécanisme d'action des solutions de chitosane [22, 23], des microsphères [24] et des nanocapsules [25], le mode d'action des CNFs n'a pas encore été abordé. Dans leur

article de revue, Martinez-Camacho *et al*. [26] ont souligné que très peu d'études se sont penchées sur les propriétés antimicrobiennes des nanofibres de chitosane (CNFs) et que des études plus approfondies dans ce domaine seraient d'une grande utilité afin d'envisager des applications potentielles de ces nanomatériaux bioactifs. Une étude cytologique de l'effet des CNFs sur la membrane bactérienne est donc nécessaire afin de mieux comprendre leur mécanisme d'action.

Le présent travail de recherche étudie les propriétés antibactériennes des CNFs obtenues par électrofilage aussi bien *in vitro* que dans des conditions réelles, lorsque les CNFs sont directement électrofilées sur un emballage alimentaire. Le mécanisme d'action antimicrobien des CNFs ainsi que leur effet sur la membrane plasmique bactérienne sont alors examinés. Cette étude évalue également l'efficacité antibactérienne des nanofibres de chitosane (CNFs) dans le maintien de la qualité microbiologique et la sécurité sanitaire de la viande rouge fraîche. Au mieux de nos connaissances, cette investigation est la première à étudier le potentiel antimicrobien des CNFs sur des aliments réels et à examiner leur capacité comme partie intégrante d'un film d'emballage à prévenir l'altération et par conséquent prolonger la durée de vie des aliments. En outre, en plus de réduire les intoxications et le gaspillage alimentaires, l'allongement de la DLC constitue un argument de vente concurrentiel recherché par tout producteur ou transformateur agroalimentaire (hypothèse validée par une étude de marché).

Organisation de la thèse

Cette thèse est basée sur trois articles qui ont été publiés ou soumis à des journaux scientifiques. Ce manuscrit est divisé en différents chapitres qui sont organisés comme suit:

- Le chapitre 2 fournit une revue de littérature et les frontières des connaissances en ce qui a trait au chitosane en général et aux CNFs plus particulièrement.
- Le chapitre 3 établit les objectifs de recherche et la cohérence des articles.
- Les chapitres 4, 5, et 6 présentent les trois articles et décrivent les principales réalisations et contributions de ce travail de recherche.
- Le chapitre 7 présente la discussion générale des principaux résultats.

Le chapitre 8 est consacré aux conclusions ainsi que les perspectives et recommandations pour les travaux futurs.

CHAPITRE 2 REVUE DE LITTÉRATURE

2.1 La chitine

La chitine est un polymère naturel produit par diverses espèces du règne animal. Naturellement présente dans la cuticule des arthropodes et arachnides et dans l'exosquelette des crustacés, elle est également présente dans l'endosquelette des mollusques et céphalopodes comme les seiches, pieuvres/poulpes et calamars [27]. Ainsi, la chitine constitue l'élément structurel de soutien des téguments de ces organismes vivants, comme le montre la Figure 2.1. Deuxième polysaccharide le plus abondant dans la nature après la cellulose, la chitine fut isolée pour la première fois par Henri Braconnot en 1811 à partir de champignons sous le nom de *fungine* [28]. En 1821, Auguste Odier isola de la chitine à partir de la carapace d'insectes et lui donna le nom de chitine, du grec *chiton*, qui signifie « tunique » [29].

Dans d'autres règnes biologiques, la chitine est également retrouvée dans la paroi cellulaire des champignons, de certaines levures, algues et bactéries [1]. Une activité enzymatique chitine synthétase (EC. 2.4.1.16) est d'ailleurs exprimée par la levure *Saccharomyces cerevisiae*. Cet organisme unicellulaire est donc capable de produire de la chitine intracellulaire [30]. Récemment, certaines espèces de champignons ont même été utilisées pour la production de la chitine à l'échelle industrielle [31, 32]. Néanmoins, leur contribution semble négligeable par rapport à celle des crustacés. Selon Jeuniaux *et al*. [33], la production mondiale de chitine par les crustacés dans les écosystèmes marins est estimée à 2.3 milliards de tonnes/an, et près de 90 % de la production totale est attribuée aux organismes pélagiques, aux crustacés, au zooplancton et au krill. De façon générale, on parle de structures chitineuses car la chitine est toujours associée à d'autres éléments de structure comme les protéines, les lipides et les minéraux (carbonate de calcium).

L'inconvénient majeur de la chitine, et probablement la raison qui empêche son utilisation pour diverses applications est sans doute liée à son insolubilité dans la plupart des solvants organiques communs. Pour cette raison, un traitement de déacétylation partielle est appliqué à la chitine afin de la rendre plus soluble. Le produit de cette conversion est appelé chitosane.

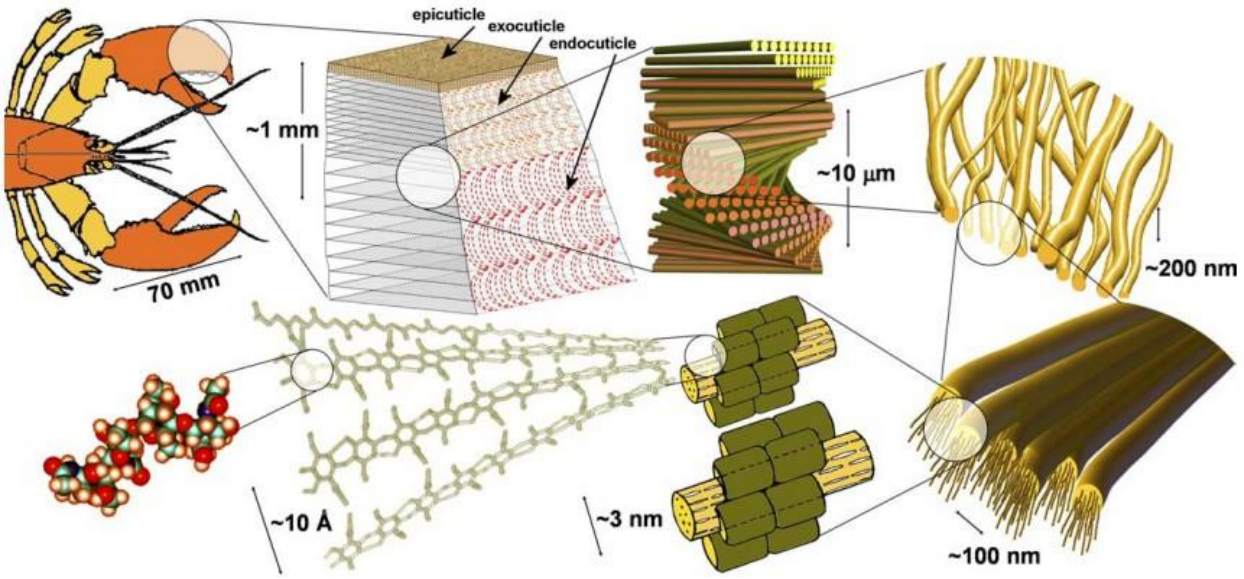

Figure 2.1: Structure de la cuticule d'un crabe et localisation de la chitine [34].

2.2 Le chitosane

2.2.1 Généralités

Le chitosane est donc le dérivé déacétylé de la chitine. Sa découverte est attribuée à Rouget (1859) [35], qui en chauffant de la chitine en présence de potasse, remarqua que le produit était soluble dans des solutions aqueuses acides [1]. Le chitosane est rarement présent dans la nature. On le retrouve dans la cuticule de certains insectes, dans le mycélium d'une classe de champignons microscopiques, les zygomycètes, dans certaines algues (*Chlorella* sp.), protozoaires, bactéries et levures [36]. Des travaux récents rapportent que certains mycètes comme *Aspergilus niger* peuvent constituer une source alternative de chitosane [37]. Berger *et. al* [32] ont également obtenu du chitosane à partir de la bioconversion de déchets agroindustriels en utilisant les souches *Rhizopus arrhizus* et *Cunninghamella elegans*. Cependant, la production industrielle de chitosane est majoritairement issue de la valorisation des sous-produits de l'industrie de la pêche.

Le procédé industriel de production du chitosane à partir de la chitine est résumé dans la Figure 2.2. Les coquilles de crustacés sont d'abord broyées puis les protéines et les pigments naturels sont extraits avec une solution d'hydroxyde de sodium. La déprotéinisation peut également être

effectuée par voie enzymatique. De l'acide chlorhydrique est ensuite utilisé pour déminéraliser (décalcifier) les coquilles et éliminer le carbonate de calcium complexé à la chitine. De l'hydroxyde de sodium ou de potassium chauffé est utilisé pour la déacétylation de la chitine. Du chitosane est alors obtenu. La durée et la température de la réaction chimique de déacétylation déterminent la pureté ainsi que la distribution du poids moléculaire du chitosane résultant [1]. En effet, ces conditions (température élevée, base forte) sont favorables à la dépolymérisation du chitosane. D'autres alternatives ont donc été développées afin de limiter sa dégradation. Il s'agit de la déacétylation par traitement thermomécanique (avec des conditions contrôlées de pression atmosphérique, température, temps et concentration en réactifs chimiques) ou encore la déacétylation par autoclavage. La déacétylation enzymatique a également été proposée afin de limiter la dépolymérisation du chitosane [38], cependant, l'utilisation d'enzymes à l'échelle industrielle n'est pas chose courante en raison des coûts et quantités élevés associés à ce type de procédé [30].

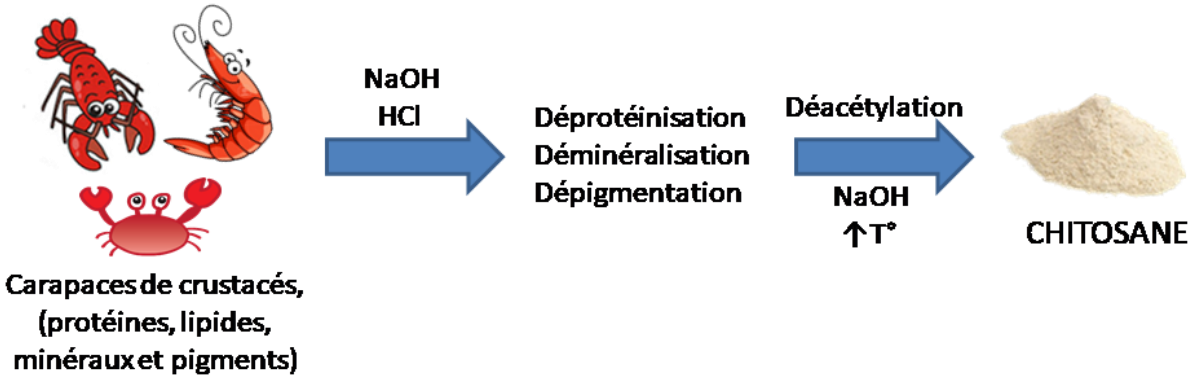

Figure 2.2: Procédé de production du chitosane à partir de l'extraction et de la déacétylation de la chitine.

La structure chimique du chitosane est très semblable à celle de la chitine et de la cellulose (Figure 2.3). La seule différence réside dans les groupements fonctionnels en position C-2; acétamide pour la chitine, amine pour le chitosane et hydroxyle pour la cellulose. Comme la chitine, le chitosane est un hétéropolysaccharide, polymère du 2-acétamido-2-désoxy-β-D-glucose et du 2-désoxy-β-D-glucopyranose, reliés ensembles par une liaison osidique β-(1→4) (Figure 2.3). Structurellement, le chitosane (et aussi la chitine) est constitué par la succession des deux unités *N*-acétyl-D-glucosamine (GlcNAc) et glucosamine (GlcN), distribuées de façon aléatoire et dont la proportion en % détermine le degré de déacétylation (DDA) du chitosane.

Ainsi, le chitosane est caractérisé par le DDA, le poids moléculaire (PM), la distribution de poids moléculaire et la viscosité intrinsèque. Outre la proportion des deux unités GlcNAc et GlcN, leur répartition spatiale le long de la chaîne moléculaire va également influer sur les propriétés physicochimiques et biologiques du chitosane.

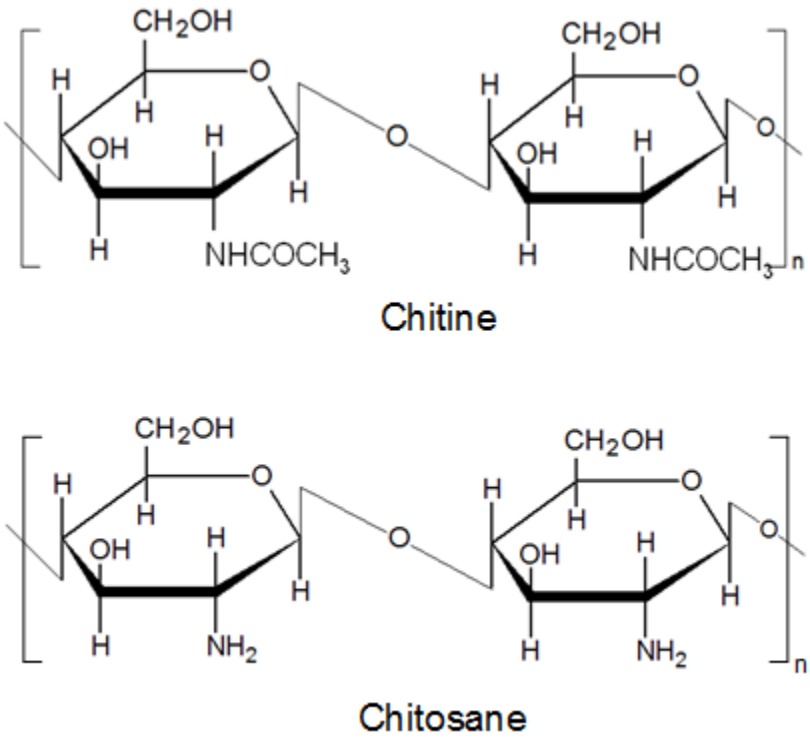

Figure 2.3. Structures chimiques de la chitine et du chitosane.

L'appellation chitosane regroupe une famille de polymères avec des grades variables de DDA, poids moléculaire, distribution de poids moléculaire et viscosité, plutôt qu'un polymère pur et unique [30]. Contrairement aux autres polysaccharides présents dans la nature et qui sont soit neutres (cellulose, amidon, dextrane, agar, gomme Arabique, gomme de caroube), ou chargés négativement (pectine, alginate, carraghénanes, xanthane), le chitosane lui est le seul polymère d'origine naturelle à être chargé positivement. Ce caractère polycationique unique lui confère bon nombre de propriétés physicochimiques et biologiques dont certaines seront discutées dans ce chapitre.

Contrairement à la chitine, le chitosane possède une structure semicristalline beaucoup moins ordonnée. De plus, la disponibilité des groupements amine ($-NH_2$) lui confère une bien meilleure

solubilité sous certaines conditions. En effet, ces fonctions deviennent protonées ($-NH_3^+$), suivant l'équilibre acido-basique illustré dans la réaction ci-dessous. Le chitosane, sous sa forme polycationique est alors soluble dans les solvants faiblement acides, lorsque le pH du milieu est en deçà de son p*K*a (6.2-6.5) [1]. Au-delà, le chitosane perd sa densité de charge positive et de ce fait les répulsions électrostatiques, causant ainsi sa précipitation. La Figure 2.4 montre la protonation – déprotonation des fonctions amines du chitosane. Par conséquent, le chitosane est un polymère fortement dépendant du pH du milieu qui le contient. Il est donc insoluble dans l'eau, dans les solvants alcalins et la plupart des solvants organiques neutres incluant l'éthanol et l'acétone. Cependant, des sels de chitosane solubles dans des solutions aqueuses peuvent être formés par neutralisation en présence d'un acide [36, 39-41]. Ce dernier peut être un acide inorganique (acide chlorhydrique) ou un acide organique (acide acétique, lactique, citrique, malique, succinique, formique, etc.).

$$R - NH_3^+ + H_2O <==> R - NH_2 + H_3O^+$$

$$K_a = [R - NH_2]\,[H_3O^+] / [R - NH_3^+]$$

$$pK_a = - \log K_a$$

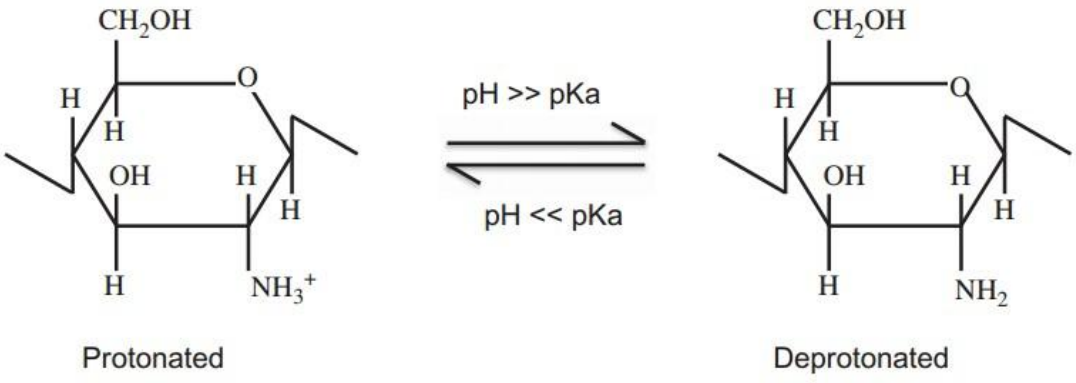

Figure 2.4: Protonation/déprotonation du chitosane dépendamment du pH du milieu [42].

2.2.1.1 Degré de déacétylation (DDA)

Le degré de déacétylation (DDA) est un paramètre clé pour la caractérisation du chitosane. Tel que mentionné précédemment, la déacétylation de la chitine donne lieu à du chitosane. D'ailleurs, la chitine et le chitosane sont considérés comme étant deux copolymères ayant des structures chimiques identiques et constituées d'unités *N*-acétyl-D-glucosamine (GlcNAc) et glucosamine (GlcN), dont la proportion détermine le degré de déacétylation (DDA). Comme le traitement de

déacétylation est presque toujours incomplet, le chitosane est considéré comme étant le dérivé partiellement déacétylé de la chitine. Théoriquement, lorsque le rapport GlcNAc/GlcN est supérieur à 50 %, le copolymère en question est la chitine et lorsque ce rapport est inférieur à 50 % on a alors à faire à du chitosane [1, 27, 38]. Mais en pratique, la distinction entre la chitine et le chitosane est basée sur la solubilité de ces deux matériaux dans une solution aqueuse d'acide acétique. Pour preuve, une chitine partiellement déacétylée à 50 % n'est pas systématiquement soluble dans une solution d'acide acétique et ne peut donc pas être considérée comme du chitosane. Par ailleurs, il a été établi que l'on peut parler de chitosane à partir d'un DDA de 60 % et non de 50 %. Une autre définition consiste donc à dire que si le matériau (chitine ou chitosane) est soluble dans de l'acide acétique (ex. 1 % v/v), on parle alors de chitosane et le cas échéant, il s'agit de chitine [35, 43].

Le DDA et la répartition des fonctions amines déacétylées influencent de façon drastique les propriétés macromoléculaires des chaînes polymériques ainsi que leur comportement en solution, à savoir la solubilité du chitosane, la flexibilité/rigidité des chaînes, la conformation des chaînes et la viscosité des solutions de chitosane. Le DDA peut être déterminé par diverses techniques chimiques ou analytiques telles que le titrage potentiométrique, l'analyse élémentaire, la spectroscopie infrarouge à transformée de Fourier (FTIR) ou encore la résonnance magnétique nucléaire (RMN) [44, 45].

2.2.1.2 Poids moléculaire (PM)

Le poids moléculaire (PM) est l'autre paramètre important dans la caractérisation du chitosane. Il est fortement affecté par les conditions du procédé de fabrication et de déacétylation et influence à son tour de façon significative les propriétés physicochimiques (comme la solubilité), les propriétés rhéologiques (à savoir la viscosité), mais encore les propriétés biologiques du chitosane (notamment l'activité antimicrobienne) [46-51]. Ainsi, dépendamment des conditions opératoires (durée du traitement chimique, nature et concentration des réactifs, conditions atmosphériques, etc.), le chitosane peut subir une dépolymérisation, réaction secondaire qui se traduit par une diminution du PM. Afin d'y remédier, des conditions du procédé d'extraction et de déacétylation moins agressives et plus contrôlées sont actuellement employées. La déacétylation enzymatique est aussi une alternative intéressante pour limiter la dégradation du chitosane et sa dépolymérisation. Du fait de la spécificité et de la régiosélectivité des enzymes

employées comme la trypsine et/ou la chitosanase, il est possible d'atteindre des distributions de PM étroites et des longueurs de chaînes contrôlées [52-54]. Cependant, en raison des coûts élevés que représente l'utilisation des enzymes à l'échelle industrielle, les grades de chitosanes obtenus par déacétylation enzymatique sont aujourd'hui encore bien plus chers que les grades conventionnels (obtenus par déacétylation chimique).

Comme pour les autres polymères (synthétiques), le PM est un paramètre extrêmement important dans les procédés de mise en forme du chitosane. Il est relié à la longueur des chaînes polymériques et est exprimé en g/mol (Da) ou en kg/mol (kDa). Le plus souvent, le PM du chitosane se décline en poids moléculaire moyen en nombre (M_n), poids moléculaire moyen en masse (M_w), ou encore en poids moléculaire moyen en viscosité (M_v). Généralement, Le PM du chitosane varie entre 100 et 1500 kDa [1, 30, 55]. Cependant, des grades de chitosane ayant des PM bien plus bas (4-50 kDa) sont disponibles commercialement. Certains vont considérer qu'en bas d'un degré de polymérisation de l'ordre de 30, on parle plutôt d'oligomères de chitosane ou chitooligosaccharides [30]. Différentes techniques permettent de mesurer le PM du chitosane. Les plus utilisées étant la viscosimétrie et la chromatographie par perméation de gel (CPG) ou chromatographie d'exclusion stérique [44, 45].

2.2.1.3 Modification chimique du chitosane

Un des inconvénients majeurs pouvant limiter l'utilisation du chitosane dans certains domaines biologiques est sa dépendance envers les milieux acides. Pour y remédier, une des stratégies utilisées est l'incorporation de chaînes d'alcanes aux groupements amines de la molécule. À cet effet, différentes modifications ou substitutions ont été proposées, parmi lesquelles la quaternisation (Figure 2.5) ou encore la carboxylation [56-58]. La quaternisation est basée sur l'introduction par le greffage chimique de chaînes alkyles sur les fonctions $–NH_2$ en position –C2 de la molécule, tandis que la carboxylation consiste à faire réagir les fonctions amine avec un groupement carbonyle. L'objectif de ces stratégies, l'une comme l'autre, était de conférer au chitosane des charges positives permanentes, d'une part pour renforcer son action antibactérienne, indépendamment du pH du milieu, et d'autre part améliorer sa solubilité (dans l'eau) [59-62]. Les réactions de modification chimique du chitosane ainsi que les avantages et limites de ces stratégies sont discutés dans un chapitre de livre consacré aux enrobages antibactériens à base de chitosane [63].

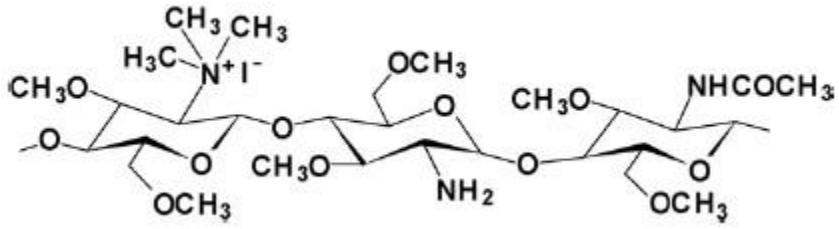

Figure 2.5: Structure chimique du chitosane quaternisé, le dérivé alkylé du chitosane [64].

2.2.1.4 Applications

Les activités biologiques uniques du chitosane, son origine naturelle, sa fonctionnalité et sa disponibilité ainsi que le large éventail de formes physiques obtenues à l'aide de procédés technologiques appropriés, sont les principales raisons de ses multiples applications [65-69]. De façon générale, les applications les plus courantes du chitosane incluent: l'administration et la libération contrôlée de médicaments en pharmaceutique [69-71]; les échafaudages et cultures de cellules en ingénierie tissulaire et cellulaire [72] et les pansements antibactériens [73] dans le domaine biomédical; le traitement des eaux [19]; la cosmétologie [74, 75]; l'agriculture [76-78]; l'industrie agroalimentaire [79-82] et l'emballage alimentaire [83-85]. Certaines de ces applications sont énumérées dans le Tableau 2.1. Mentionnons que la quasi-totalité des applications énumérées dans le tableau sont encore au stade de recherche, bien que certaines commencent actuellement à émerger. Ajoutons toutefois que plusieurs brevets en lien avec des applications commerciales ont été décelés, c'est le cas du brevet sur l'utilisation du chitosane comme clarifiant dans les vins [86], il y a aussi des brevets relatifs à l'utilisation du chitosane comme additif ou enrobage alimentaire [87, 88], ou encore les nombreux brevets de DuPont quant à l'utilisation de films à base de chitosane dans le textile pour l'absorption de colorants [89-91].

À l'état brut, le chitosane a été utilisé dans les soins de santé comme agent amincissant pour ses propriétés hypocholestérolémiantes et de rétention des graisses. En ce qui concerne l'industrie alimentaire, le chitosane a été largement utilisé comme additif en raison de sa non toxicité, comme émulsifiant ou stabilisant, comme épaississant et comme agent gélifiant pour stabiliser les aliments (e.g.: les émulsions) [92, 93]. On connait également au chitosane une application dans la clarification des vins et du jus de pomme pour l'obtention d'un produit limpide. En effet, la

pectine étant aussi un polysaccharide mais chargé négativement, la formation de complexes chitosane-pectine permet de précipiter les particules de pectine en suspension [1].

En raison de ses propriétés filmogènes, le chitosane a souvent été utilisé comme un agent d'enrobage alimentaire [77, 94, 95]. Les films de chitosane ont été prouvés efficaces pour absorber la transpiration des aliments dits « qui respirent » et pour inhiber le développement des champignons, ce qui retarde la maturation et le pourrissement des fruits et légumes, réduisant ainsi le gaspillage des aliments [23, 80, 96-98].

Le chitosane a également été utilisé comme agent antioxydant et comme conservateur alimentaire. Selon Darmadji *et al*. [99], l'ajout de 1% de chitosane peut suffire à ralentir l'oxydation des acides gras mono- (e.g.: acide oléique) ou polyinsaturés (e.g.: ω3), réaction provocant une saveur désagréable et une rancidité de certains aliments dont le poisson, les fruits de mer, les noix et les huiles. De même, les auteurs ont rapporté qu'une concentration de 0.01 % en chitosane suffit à inhiber la croissance des bactéries de la flore d'altération comme *Escherichia coli, Staphylococcus aureus, Bacillus subtilis* et *Pseudomonas aeruginosa*.

Enfin, il est à noter que jusqu'à présent, aucun effet indésirable des enductions/enrobages et des films de chitosane n'a été rapporté en ce qui a trait aux propriétés organoleptiques (goût, odeur, saveur, couleur et texture) des produits alimentaires comme les fruits, les viandes ou les œufs en contact avec le chitosane [1, 85, 100-102]. Celui-ci est considéré comme un matériau assez inerte et qui ne modifie pas significativement les propriétés organoleptiques des aliments testés. Cependant, l'effet potentiel sur la biodisponibilité de certains micronutriments et sur la qualité nutritionnelle des produits avec lesquels il est en contact reste peu étudié.

Tableau 2.1: Applications industrielles, produits et revêtements à base de chitosane.

Segment/marché	Produits à base de CS	Description	Référence
Soins de Santé	Pâte à dent, rince bouche, gomme à mâcher	Rince bouche sans alcool, prévient la formation de plaque et de caries	[103, 104]
	Produit amincissant	Capteur de graisses, effet hypocholestérolémiant	[66, 103]
	Solutions antimicrobiennes	Produit désinfectant	[103]
Cosmétique	Champoings, soins et sprays pour cheveux, gels et lotions coiffantes	Effet hydratant, élimine l'effet électrostatique, améliore la souplesse, la douceur et fortifie le cheveu	[47, 66, 74, 103, 105]
	Soins de la peau, lotions et crèmes	Propriétés d'hydratation et de remplissage	
	Maquillage et autres formulations cosmétiques	Vernis à ongles, fards à paupières, rouges à lèvres, déodorants	
Filtration de l'eau	Membranes non-tissées à base de chitosane, microsphères	Chélation des ions métalliques, métaux lourds et bactéries dans le traitement des eaux usées. Résines échangeuses d'ions pour utilisation en chromatographie	[6, 106]
Industrie du textile	Fibres filées à base de chitosane	Textiles innovants propriétés antimicrobiennes et anti odeurs	[66, 107]
Papeterie Photographie	Enrobages à base de chitosane (films et solutions)	Hautes propriétés (brillance), résistance à l'abrasion	[66]
Transformation alimentaire	Chitosan à l'état brut (poudres et flocons)	Additif et conservateur, encapsulation d'odeurs, arômes, vitamines et antioxydants	[108, 109]
Emballages alimentaires	Solutions, films et nanostructures de chitosane	Emballages alimentaires antimicrobiens et comestibles	[110]
Agriculture	Géomembranes, sprays et enductions à base de chitosane (solutions)	Stimulateur du système de défense des plantes, biopesticide et biofongicide (lutte biologique)	[1, 111]
Biomédical	Échafaudages à base de nanofibers, nanoparticles de chitosane	Culture de cellules cartilagineuses et osseuses, peaux artificielles (greffes), complexes chitosane-ADN pour thérapie génique, délivrance de gène, transfection et traitement des allergies	[11, 112, 113]
Pharmaceutique	Micro et nanostructures à base de chitosane	Libération contrôlée de médicaments, pansements antibactériens, encapsulation de molécules actives, d'intérêt et autres substances médicinales components	[114-116]

2.2.2 Propriétés biologiques

2.2.2.1 Innocuité / toxicité

Le chitosane est connu pour son innocuité et sa faible cytotoxicité a été prouvée par diverses études cliniques *in vitro* mais aussi *in vivo* chez l'animal et l'homme. Cette particularité rend possible l'utilisation du chitosane dans diverses applications allant du domaine alimentaire au pharmaceutique, en passant par le biomédical, ce qui explique la grande versatilité de ce biopolymère. Une étude menée chez l'homme et portant sur l'effet chélatant sur les graisses après administration de 4.5 g/jour de chitosane par voie orale (DDA et PM non déterminés) a démontré l'absence d'une quelconque toxicité ou effets indésirables [117, 118]. Arai *et al*. [119] rapportent que la dose létale 50 (DL_{50}) du chitosane est presque comparable à celle du sucrose et du sel, celle-ci étant estimée à 16 g/jour. kg de masse corporelle (administration orale chez la souris). Une autre étude montre l'absence de toxicité suite à l'administration orale de 100 mg/kg de chitosane (80 kDa, 80 % DDA) chez la souris. D'autres études reportent l'innocuité de l'administration orale et intraveineuse de doses de chitosane de 1 g/jour. kg et 4.5 g/jour. kg, chez les lapins et les poulets, respectivement [1]. Cependant, ce qu'il est important de souligner, c'est l'absence d'un élément crucial manquant dans les études mentionnées ici; il s'agit du facteur temps. Car en effet, même si ces études ne mentionnent aucun effet toxique dose-dépendant, il est toutefois important de tenir compte de la durée d'exposition (orale, cutanée, intraveineuse, parentérale, etc.).

Des études ont démontré l'innocuité du chitosane chez l'homme après une exposition longue durée (12 semaines) par ingestion, outre de légères diarrhées ou constipations chez une faible portion des individus participants [120]. En revanche, des mises en garde existent quant à l'exposition prolongée au chitosane et Tanaka *et al*. [121] préviennent qu'une attention particulière doit être accordée à ce point. En effet, lorsque du chitosane est administré par voie orale ou parentérale à des souris de façon prolongée, des effets néfastes ont été observés. Les chercheurs ont constaté une diminution significative du poids des souris, suivie par une perturbation de la flore intestinale, des carences dues à la mauvaise absorption de certaines vitamines liposolubles ou à la désorption du contenu minéral dans les os, mais encore la non absorption de certaines substances médicamenteuses.

Il est souvent rapporté que le degré de pureté du chitosane, le DDA et le PM ainsi que les traitements de modification/substitution chimique peuvent influencer son profile toxicologique [122, 123]. Il est donc de rigueur de s'assurer de la qualité du chitosane utilisé et du respect strict des recommandations réglementaires lors des essais cliniques. Un effort de standardisation est donc requis car les études sont souvent pertinemment incomparables entre elles, d'autant plus que des grades de chitosane ayants des propriétés bien définies sont actuellement disponibles.

2.2.2.2 Biocompatibilité

Le chitosane est considéré comme un matériau biocompatible car sa présence et son interaction dans les tissus et cellules des organismes vivants entraînent de très faibles réactions inflammatoires et de rejet. Ses propriétés immunologiques sont donc très limitées car le chitosane est reconnu par les organismes comme un élément du soi, un agent non étranger, non immunogène, n'ayant pas de pouvoir antigénique. Le chitosane est donc très bien toléré par les tissus vivants incluant l'épiderme, les tissus osseux, l'épithélium nasal, les membranes oculaires, les systèmes neurologique, respiratoire, cardiovasculaire, hépatique, urinaire, digestif et génétique (ADN) [75, 122, 124, 125]. Il est d'ailleurs naturellement résorbable avec des cinétiques contrôlées [1, 126]. Les études actuellement disponibles ont montré que le chitosane est un matériau sans danger pour la santé. Cependant, des contre-indications proscrivent son utilisation dans les cas d'allergies aux fruits de mer [127].

2.2.2.3 Biodégradabilité

Actuellement, le terme « biodégradable » est un qualificatif parfois utilisé à tort et à travers pour désigner des matériaux qui ne le sont pas toujours. La biodégradabilité d'un matériau est définie par la capacité de ce dernier à être dégradé sous l'action enzymatique microbienne [21, 128]. La biodégradabilité implique inévitablement une première étape de biofragmentation du matériau en petites molécules assimilables par les cellules microbiennes pour y être ensuite décomposées en eau, en dioxyde de carbone, en méthane, en composés non organiques et en biomasse. Étant donné que certaines espèces microbiennes dont une catégorie de mycètes et de bactéries sont connues pour leur production de chitosane, il va de soi que ces microorganismes sont également capables de le dégrader car ils possèdent des mécanismes de reconnaissance de soi et par conséquent les enzymes nécessaires à sa synthèse et à sa dégradation.

On sait, par ailleurs que le chitosane et la chitine sont susceptibles à la dégradation sous l'action d'une large variété d'enzymes spécifiques telles les cellulases ou les hémicellulases, [129] les chitinases [130], les lipases, les glucanases et les chitosanases [131], ou non spécifiques comme le lysozyme (présent dans la salive, l'œuf et le lait) et les protéases (papaïne et pronase) [132]. Une des enzymes utilisées pour la fragmentation du chitosane et la production d'oligomères de chitosane est la chitosanase EC 3.2.1.132 (ou chitosane *N*-acétyl-glucosamine hydrolase) [30, 36]. Cette enzyme catalysant l'endohydrolyse de la liaison osidique *ß*-(1→4) entre les unités D-glucosamine (GlcNac) clive de façon spécifique le chitosane mais pas la chitine [131, 133] (Figure 2.6). Par ailleurs, le chitosane est également sensible à l'hydrolyse acide et à la dégradation oxydo-réductive. Étant un matériau biodégradable, toute la difficulté avec le chitosane réside dans la conception de systèmes stables pour une utilisation première optimale suivie d'une dégradation jusqu'à assimilation totale en fin de vie du produit.

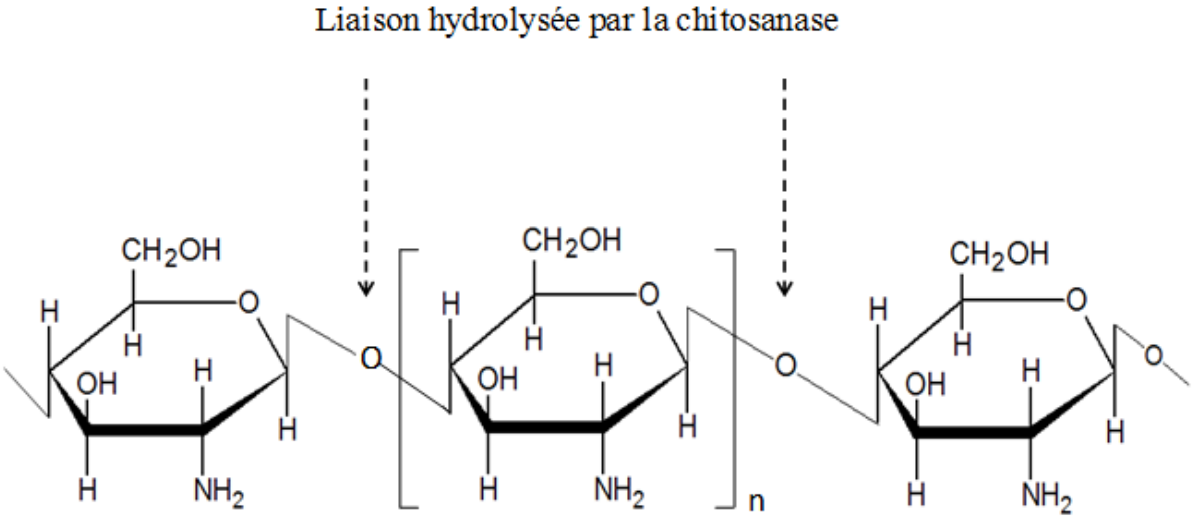

Figure 2.6: Clivage du chitosane par une enzyme spécifique, la chitosanase.

2.2.2.4 Autres propriétés

Le chitosane possède une variété de propriétés biologiques exceptionnelles qui sont à l'origine de nombreuses recherches publiées chaque année et qui sont pour la plupart attribuées à sa bioactivité, elle-même attribuée aux groupements fonctionnels $-NH_3^+$. La bioactivité est définie comme étant l'effet qu'a une molécule sur un organisme ou un tissu suite à son interaction avec l'un ou l'autre de ces derniers [1, 126].

Outre ses propriétés biologiques, le chitosane exhibe également certaines propriétés physicochimiques qui émanent des fonctions amines mais aussi des liaisons glycosidiques β-(1→4). Ces fonctions confèrent au chitosane son comportement semi-rigide et sont à l'origine de son pouvoir épaississant, *i.e.* sa capacité à atteindre des viscosités élevées en solution. Ces mêmes fonctions glycosidiques sont aussi à l'origine des propriétés filmogènes du chitosane, au même titre que beaucoup d'autres polysaccharides.

Les propriétés antimicrobiennes du chitosane ne sont plus à démontrer. En effet, il inhibe la croissance de nombreux virus, spores, bactéries, levures, moisissures et parasites. Il aurait même une activité anti-toxinogène contre certaines mycotoxines produites par certains mycètes, et dont certaines sont connues pour leur effet cancérigène. Le chitosane réduit la synthèse de l'aflatoxine mortelle d'*Aspergillus flavus*, tout en inhibant la croissance de cette souche fongique [1]. Les propriétés antibactériennes du chitosane seront discutées dans la section 3.2.3.

Le chitosane possède une puissante activité anti-oxydante, il empêche ainsi la formation d'espèces réactives de l'oxygène comme les radicaux libres, ce qui lui confère des propriétés de ralentissement du processus de vieillissement, parfois responsable de l'apparition de certaines formes de cancers. Le chitosane possède aussi des propriétés antitumorales et antiprolifératives [134].

Le chitosane est bio-résorbable dans les tissus biologiques, il possède des propriétés hémostatique et anti-thrombogénique. Ces propriétés ont souvent fait du chitosane un matériau de choix dans le domaine pharmaceutique et biomédical. Il a été utilisé comme excipient ou pour encapsuler des molécules actives (pour une libération contrôlée) dans certains médicaments. Son caractère à la fois hémostatique et antimicrobien a été exploité pour la fabrication de pansements bioactifs ultra absorbants de certains fluides comme le sang. De plus, il ne présente aucun caractère antigénique, les réactions immunitaires, inflammatoires et de rejet sont alors très limitées.

Une des propriétés du chitosane ayant donné lieu à une application commerciale est sa capacité à capter les graisses. Utilisé dans certains produits amincissants, le chitosane chélate les acides gras, empêchant alors leur absorption et favorisant leur élimination par voie naturelle. Le caractère polycationique du chitosane lui confère également un pouvoir hypocholestérolémiant qui est attribué à son interaction sélective avec les substances anioniques telles que les lipides.

On connait au chitosane des propriétés cicatrisantes remarquables. En effet, il augmente la vitesse de cicatrisation en stimulant la croissance cellulaire et la réparation des tissus des plaies opératoires par exemple, tout en évitant les infections microbiennes. Ces propriétés uniques ont donné lieu à des applications de plus en plus nombreuses dans le domaine biomédical [10, 11, 115, 122, 135].

2.2.3 Propriétés antibactériennes du chitosane

Les propriétés antimicrobiennes du chitosane et ses dérivés ne sont plus à prouver et plusieurs études ont démontré leur potentiel antimicrobien aussi bien *in vitro* qu'*in situ* (sur des aliments réels et les tissus biologiques). Toutefois, la majorité des travaux publiés portent sur les solutions et les films à base de chitosane [46, 66, 96, 136-141]. Le chitosane possède donc une puissante activité antimicrobienne contre un large spectre de souches bactériennes, fongiques et même contre des levures, virus et spores. Dans ce chapitre, il sera question des propriétés antibactériennes du chitosane, et de façon plus spécifique contre une large gamme de bactéries à Gram positif et à Gram négatif. Une définition des deux types bactériens est donnée un peu plus loin dans la section 2.2.3.3.

2.2.3.1 Mécanisme d'action

Pour tenter d'expliquer le mécanisme antibactérien du chitosane, trois possibles modes d'action ont été proposés dans la littérature [24, 37, 142, 143]. (1) Selon le premier mode d'action, le chitosane déstabiliserait la perméabilité membranaire en interagissant avec la paroi bactérienne *via* ses groupements fonctionnels protonés NH_3^+. Le chitosane interagirait alors avec les charges négatives des éléments constituant la paroi bactérienne. Par conséquent, il perforerait la cellule bactérienne, provoquant ainsi le relargage du contenu cytosolique et la fuite des composants intracellulaires; (2) Le second suggère que le chitosane formerait une enveloppe autour de la cellule bactérienne, empêchant ainsi les échanges avec le milieu extérieur, non seulement pour l'assimilation des nutriments mais aussi l'excrétion des toxines accumulées dans le cytoplasme de la bactérie. De plus, le chitosane déstabiliserait l'équilibre physiologique des bactéries en chélatant les oligoéléments essentiels à leur croissance; (3) Selon le troisième mode d'action, la complexation du chitosane avec l'ADN génomique (chargé négativement) serait responsable de son activité antibactérienne. L'interaction chitosane-ADN interférerait avec la réplication de

l'ADN, bloquant ainsi la transcription de l'ARN messager (ARN_m) et la traduction de certaines protéines indispensables à la croissance bactérienne. Dans ces trois cas de figure, les interactions CS-bactérie mènent inévitablement à la lyse et à la mort cellulaire. Cependant, la probabilité du dernier mode d'action suggéré a été jugée faible et celui-ci a été considéré comme étant une conséquence de l'un des deux mécanismes susmentionnés car pour atteindre le matériel génétique de la bactérie, le chitosane doit d'abord perforer la paroi.

2.2.3.2 Effet sur la membrane bactérienne

Les propriétés antibactériennes du chitosane ont été largement étudiées contre une vaste gamme de bactéries. Certains auteurs considèrent le chitosane comme agent antibactérien bactériostatique [21, 128]. Autrement dit, le chitosane serait capable d'inhiber la multiplication des bactéries sans pour autant les tuer, tandis que d'autres lui attribuent un effet bactéricide, c'est à dire la capacité de tuer les bactéries et non juste freiner leur croissance [110]. Peu de travaux sont disponibles quant à l'effet que le chitosane peut avoir sur la paroi bactérienne. Néanmoins, il semble que les interactions entre les fonctions amines protonées $–NH_3^+$ du chitosane et les motifs chargés négativement des phospholipides membranaires soient responsables de la perturbation de l'homéostasie *i.e.* l'équilibre physiologique de la cellule. Le chitosane déstabiliserait la perméabilité membranaire et causerait la fuite du contenu intracellulaire dont protéines, matériel génétique (ADN, ARN), minéraux, etc. Toutefois, la perforation de la membrane plasmique par les chaînes du chitosane n'a pas été prouvée et ce malgré le fait que la rupture de la membrane ait bien été observée [143, 144]. Enfin, le mécanisme ainsi que les voies métaboliques *via* lesquelles le chitosane est internalisé à travers la paroi pour rejoindre la membrane cytoplasmique restent à ce jour inconnus.

Raafat *et al*. [22] ont étudié le mécanisme d'action de solutions de chitosane contre la bactérie *S. aureus*. Ils ont conclu que le mode d'action du chitosane est un phénomène complexe qui n'a pas été entièrement élucidé. Néanmoins, les auteurs ont rapporté que l'acide lipoteichoïque (LTA), un glycolipide présent dans la membrane des bactéries à Gram positif pourrait être impliqué dans le mécanisme d'action du chitosane contre *S. aureus*, et ce par interaction entre les motifs $–NH_3^+$ et les charges négatives du LTA. Cependant, l'implication du LTA dans le mécanisme d'action n'explique que partiellement la sensibilité de la bactérie *S. aureus* aux solutions de chitosane. En

effet, les bactéries à Gram négatif sont dépourvues de LTA et sont tout de même sensibles à l'action du chitosane.

Récemment, Hammer *et al*. [145] ont étudié l'implication possible du lipopolysaccharide (LPS) dans le mode d'action d'un aminopeptide synthétique (AMP) NK-2 contre les bactéries à Gram négatif dont *E. coli* et *Proteus mirabilis*. Les auteurs ont conclu que l'AMP s'intercale à travers les bicouches lipidiques pour se lier au LPS. Puisque la membrane bactérienne constitue la première barrière face aux agressions externes, l'intercalation de l'AMP à travers la membrane et son interaction avec sa cible, le LPS causerait alors des lésions hétérogènes dans les membranes bactériennes. Ceci suggère que les structures intracellulaires telles que l'ADN sont les cibles secondaires de l'AMP. On pourrait alors supposer que ce mécanisme d'action puisse s'appliquer au chitosane, étant donné la présence de fonctions amines dans les deux molécules (AMP et chitosane), une piste qu'il serait pertinent d'investiguer plus en profondeur afin de mieux comprendre le mécanisme d'action du chitosane.

2.2.3.3 Sensibilité des bactéries à Gram positif versus Gram négatif

Bien que la composition des parois cellulaires des bactéries à Gram négatif et des bactéries à Gram positif soit la même, il existe néanmoins des différences au niveau structurel entre ces deux types de bactéries (Figure 2.7). La paroi cellulaire bactérienne des Gram positif (à droite) est composée de deux couches: une couche épaisse de peptidoglycane ou muréine (10 à 80 nm soit 40 % du poids sec total) recouvrant la membrane plasmique constituée d'une seule bicouche lipidique [128]. D'autre part, la paroi des bactéries à Gram négatif (à gauche) est composée de trois couches: une membrane externe constituée d'une bicouche phospholipidique riche en lipopolysaccharide (LPS) et lipoprotéines, une mince couche de peptidoglycane (entre 2 et 6 nm, soit 10 % du poids sec total) et une membrane plasmique interne, la cible. Habituellement, il est possible de distinguer et de classifier les deux types bactérien en laboratoire en faisant une coloration de Gram suite à laquelle les Gram positif prennent une couleur violacée tandis que les Gram négatif gardent une teinte rosée [146].

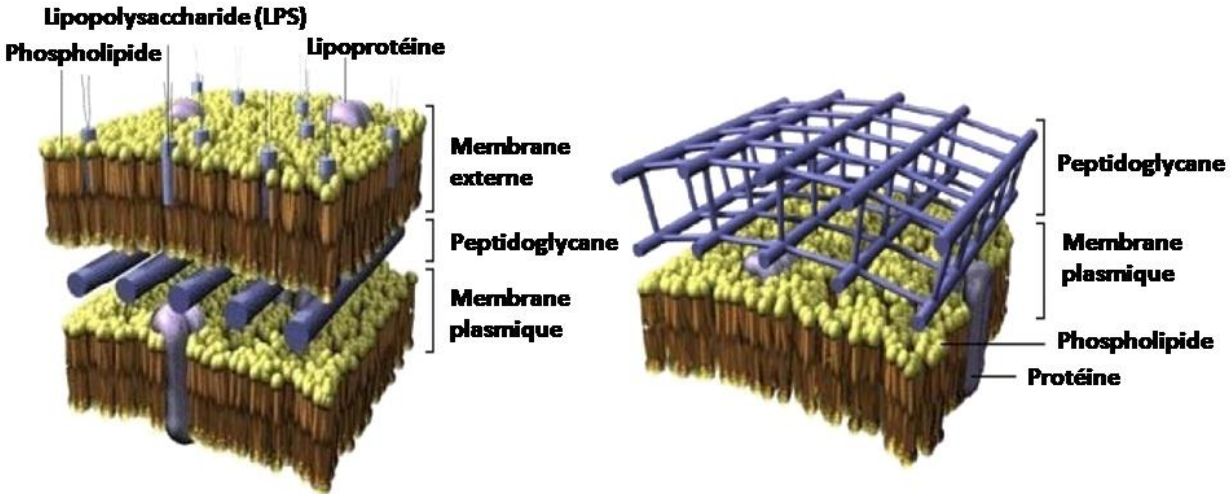

Figure 2.7: Parois bactérienne des bactéries à Gram négatif (à gauche) et des bactéries à Gram positif (à droite) [147].

L'efficacité antibactérienne du chitosane contre les bactéries à Gram négatif et les bactéries à Gram positif a suscité beaucoup de controverse dans la littérature. Cette différence de sensibilité a été attribuée aux différences structurelles de la paroi des deux types de bactéries. Certains auteurs ont clamé que les bactéries à Gram positif sont plus sensibles que les bactéries à Gram négatif du fait de la complexité moindre de leur paroi (deux couches au lieu de trois) [49, 50, 142, 148-150]. Le chitosane pourrait donc facilement se glisser à travers le réseau de peptidoglycane pour atteindre la membrane plasmique. La membrane externe des bactéries à Gram négatif agirait comme une barrière supplémentaire efficace et pourrait freiner et/ou empêcher le chitosane d'atteindre la membrane cytoplasmique. Ainsi, il a été montré que le chitosane possède généralement une activité antibactérienne plus efficace contre les bactéries à Gram positif, celles-ci étant donc plus sensibles à son action. Inversement, il a été établi que la densité de charge négative sur la paroi cellulaire des bactéries à Gram négatif est supérieure à celle de leurs consœurs Gram positif. De plus, l'hydrophilicité et la polarité des bactéries à Gram négatif sont également significativement plus élevées que celles des bactéries à Gram positif. Cette densité de charge positive et cette hydrophilicité accrues chez les bactéries à Gram négatif sont conférées par la présence de lipopolysaccharides (LPS), dont les bactéries à Gram positif sont dépourvues. Par conséquent, l'affinité, l'interaction et l'adsorption des chaînes de chitosane sur la membrane des bactéries à Gram négatif les rendraient plus sensibles à son action [32, 138, 144]. Une efficacité qui reste encore mitigée dans la littérature.

2.2.3.4 Paramètres influençant l'activité antibactérienne

Même si plusieurs études ont montré que les propriétés antimicrobiennes du chitosane dépendent de façon significative du poids moléculaire (PM) et du degré de déacétylation (DDA), des résultats contradictoires ont été rapportés. Pour citer des exemples, Chung et Chen [20, 144] ont observé un effet bactéricide plus élevé pour des solutions de chitosane ayant un haut DDA. Inversement, Park *et al*. [51] ont rapporté que l'activité antimicrobienne du chitosane n'était pas proportionnelle à son DDA. L'effet du PM est encore plus déroutant et de nombreuses combinaisons d'hypothèses coexistent. Ainsi, Zheng et Zhu [50] ont affirmé qu'une solution de 0.25 % chitosane de faible poids moléculaire (< 5 kDa) s'est avérée plus efficace pour inhiber la croissance de la bactérie *E. coli*, tandis que, dans le cas du Gram positif *S. aureus*, une activité antibactérienne plus prononcée a été obtenue avec des PM plus élevés (305 kDa). Vraisemblablement, l'effet du PM et du DDA sur l'activité AB du CS suscite encore la controverse dans la littérature. Un point essentiel que nous nous sommes attelés à clarifier.

2.2.4 Réglementation et taille de marché

La taille du marché mondial du chitosane a été estimée à 3.19 milliards USD en 2015 et devrait connaître une croissance significative et atteindre 17.84 milliards USD d'ici 2025, selon les prévisions (Figure 2.8). D'après un récent rapport du Grand View Reasearch Inc [151], la forte demande du marché global de chitosane est attribuée à la hausse des champs d'applications dans le secteur du traitement de l'eau et des effluents (chélation des métaux lourds et autres contaminants par floculation), dans le pharmaceutique, biomédical, cosmétique et l'agroalimentaire.

Le chitosane a été approuvé comme additif alimentaire au Japon et en Corée depuis 1983 et 1995, respectivement. En Chine, il a été approuvé comme épaississant alimentaire dans les produits carnés depuis 2007 [89]. L'Italie et la Finlande l'ont également approuvé comme ingrédient alimentaire et aux États-Unis, le chitosane a reçu le statut GRAS (Generally Recognized As Safe) par la FDA (Food and Drug Administration) pour utilisation dans des applications biomédicales mais aussi en tant qu'additif dans l'alimentaire [152]. Au Canada, l'utilisation du chitosane au contact des aliments dans le domaine alimentaire n'est pas encore approuvée par Santé Canada.

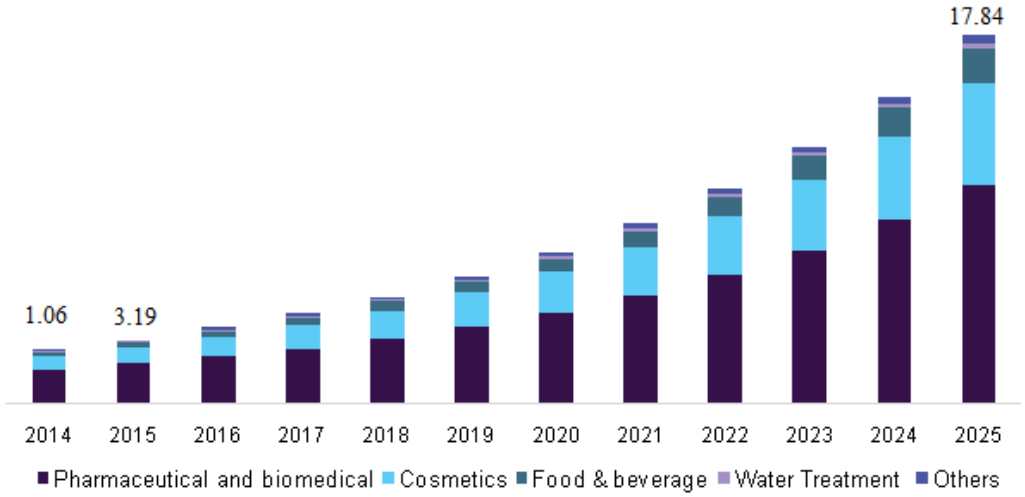

Figure 2.8: Prévisions de l'évolution du marché américain du chitosane par domaine d'application de 2014 à 2025 (milliards USD) [151].

2.3 La technologie des nanofibres

Contrairement au filage conventionnel de fibres de polymères (filage fondu) où la taille des fibres obtenues est de l'ordre de quelques microns, l'électrofilage (ou filage électrostatique) est un procédé relativement simple et peu couteux. Il permet d'obtenir des fibres d'une taille à l'échelle nanométrique avec des propriétés remarquables. Les nanofibres obtenues présentent des propriétés remarquables comme un diamètre inférieur à 100 nm, avec un rapport d'aspect de plus de 100, une grande surface spécifique (10–500 m^2/g), soit plus de trois fois la surface d'un terrain de tennis dans un gramme de nanofibres, une porosité élevée (~ 80 %) avec des diamètres de pores assez faibles (10^2–10^4 nm) [10, 113, 153].

La technologie des nanofibres est en plein essor et ce en raison de la diversité des applications potentielles, en particulier dans certains domaines délicats comme la chirurgie (fil chirurgical, greffes de peaux, tissus et organes artificiels), le biomédical (pansements, échafaudages pour l'ingénierie tissulaire et la culture de cellules) et la filtration membranaire. Ainsi, les matériaux à base de nanofibres polymériques ont très souvent été utilisés dans des applications biomédicales. Ceci peut être expliqué par le rendement du procédé d'électrofilage plus ou moins faible en comparaison avec les procédés commerciaux de mise en forme des polymères, le rendant ainsi difficilement transposable à grande échelle. Toutefois, des montages industriels commencent à voir le jour et sont aujourd'hui disponibles pour la montée en échelle (Figure 2.9).

Figure 2.9: Montage d'électrofilage de laboratoire versus un dispositif industriel (Nanospinner®) http://bageneff.com/Nanotechnology.html

2.4 Les nanofibres de chitosane

Durant les dernières décennies, des nanofibres à base de biopolymères dont le collagène, l'acide hyaluronique, la soie, la cellulose, l'alginate, la chitine et le chitosane ont vu le jour et leur utilisation ne cessent de prendre de l'ampleur [10]. Parmi les biomatériaux les plus prisés et les plus fréquemment utilisés, les CNFs peuvent être facilement obtenues par le procédé d'électrofilage. De plus, pour compenser les propriétés mécaniques médiocres du chitosane, des nanofibres combinant chitosane et autres polymères biocompatibles et biodégradables ont été préparées dans le but d'équilibrer les propriétés antibactériennes et mécaniques des nanomatériaux bioactifs ainsi obtenus [10]. Ainsi, des progrès remarquables ont été observés aussi bien dans les méthodes de préparation que dans les applications des nanofibres à base de chitosane (CNFs).

En raison de leurs propriétés biologiques en lien avec leur biodégradabilité, résorbabilité, biocompatibilité, activité antimicrobienne, faible immunogénicité, activité régénératrices, hémostatiques et de rétention des fluides, différentes méthodes ont permis de préparer des nanostructures/nanofibres à base de chitosane. Il s'agit notamment de l'électrofilage,

l'électropulvérisation, l'impression 3D, la gélification ionique, la complexation par traitement chimique, la séparation de phase ou encore l'ultrasonication [154-158]. Dans ce travail, les CNFs sont préparées par le procédé d'électrofilage.

2.4.1 Le procédé d'électrofilage

L'électrofilage est un procédé relativement simple, peu contraignant, peu coûteux et très efficace dans l'obtention de fibres à l'échelle nanométrique (5-500 nm). Il est possible, grâce à ce procédé d'obtenir des nanofibres uniformes et continues à partir d'une large gamme de polymères. Tout dispositif d'électrofilage est composé de trois éléments : une pompe propulsant la solution de polymère contenue dans une seringue au débit souhaité; un générateur de courant à haut voltage (0-50 kV) et un collecteur en métal permettant de récupérer les nanofibres. Le schéma d'un dispositif classique d'électrofilage est illustré dans la Figure 2.10.

L'électrofilage repose sur l'étirage uniaxial d'une solution viscoélastique. Un champ électrique à haut voltage est appliqué à une solution de polymère contenue dans une seringue et propulsée à un débit déterminé. Un équilibre entre les forces électrostatiques, la viscosité et la tension superficielle de la solution produit une déformation de la goutte polymère sortant de l'aiguille en une forme de cône communément appelé « cône de Taylor » du sommet duquel, un jet est éjecté, allongé et accéléré vers la contre-électrode. En 1964, Taylor a étudié la déformation d'une goutte de polymère en une forme conique lorsque celle-ci est soumise à un courant électrique [159]. Lorsque les forces électrostatiques dans le fluide arrivent à surmonter la tension superficielle, le jet est divisé en plusieurs filaments chargés, les nanofibres. Ces nanofibres se déplacent vers le collecteur tandis que le solvant s'évapore. Enfin les nanofibres sont récupérées sur la plaque collectrice. Une image en microscopie électronique à balayage montre la morphologie des nanofibres obtenues par électrofilage (Figure 2.11).

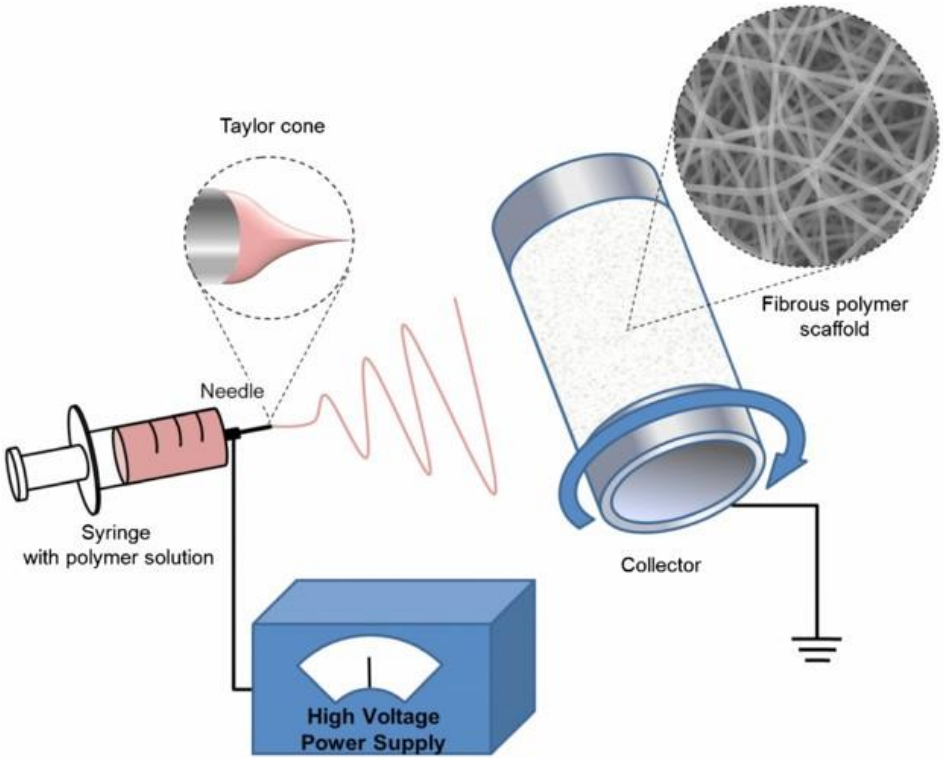

Figure 2.10: Schéma représentatif du montage d'électrofilage [160].

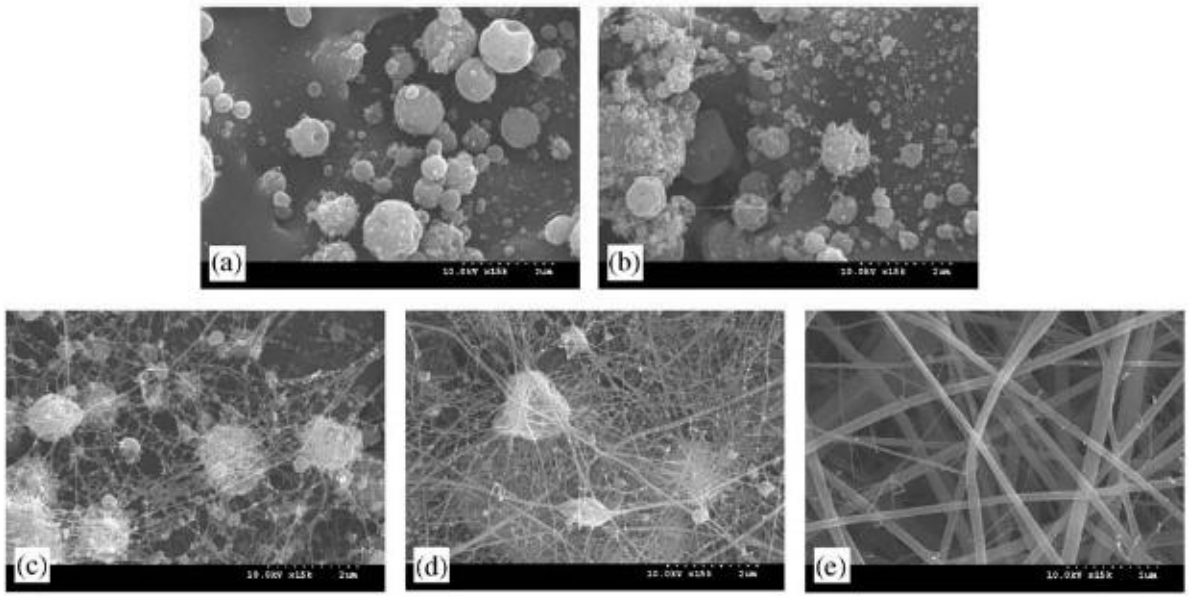

Figure 2.11: Morphologie en MEB de nanofibres de chitosane obtenues par électrofilage et solubilisé dans de l'acide acétique (AcOH). Concentrations en AcOH (% v/v) : (a) : 10 %, (b) : 30 %, (c) : 50 %, (d) : 70 % et (e) : 90 % [7].

2.4.2 Électrofilage du chitosane

L'électrofilage des polymères (synthétiques ou naturels) est un procédé prometteur en vue de l'obtention de nanofibres ultrafines, comparables à des fibres de collagènes. L'électrofilage du chitosane a suscité beaucoup d'intérêt au court des dix dernières années. Les premières tentatives d'électrofilage de ce biopolymère polycationique ont échoué car l'électrofilage du chitosane est une tâche complexe. Les principales raisons de la mauvaise électrofilabilité du chitosane pourraient être les suivantes: (i) lorsqu'il est solubilisé à un pH inférieur à son p*K*a, le chitosane agit comme un polyélectrolyte cationique et ses fonctions amines protonées favorisent les forces de répulsions électrostatiques. Si cette propriété permet sa solubilisation et donne lieu à de nombreuses biofonctionnalités intéressantes (antibactériennes, antifongiques, antiprolifératives, antioxydantes, antitumorales), il en est autrement pour la flexibilité des chaînes. (ii) En effet, les répulsions électrostatiques sont importantes pour sa solubilisation mais elles rendent, néanmoins les chaînes du chitosane rigides et leur contorsion et enchevêtrement difficile. (iii) De plus, la formation de liaisons hydrogènes inter- et intra-chaînes réduisent également la flexibilité des macromolécules et augmentent considérablement la viscosité des solutions. Par conséquent, il en résulte un enchevêtrement et une flexibilité des chaînes insuffisants pour initier le filament de chitosane et permettre son élongation en vue de l'obtention des nanofibres [4, 161].

Par la suite, certains chercheurs sont parvenus à obtenir avec succès des nanofibres de chitosane parfaitement lisses et homogènes [3-5, 8, 9, 126]. Certains sont même parvenus à préparer des nanofibres de chitosane pur, sans ajout d'agent de co-électrofilage [7, 161, 162]. Toutefois, si on approfondit la recherche et que l'on s'intéresse aux types de solvants employés, on s'aperçoit vite que dans la plupart des études, les solvants utilisés sont très agressifs voire hautement toxiques. C'est le cas, entre autres de l'acide trifluoroacétique (TFA), du dichlorométhane (DCM) [6, 10, 163-165], le 1,1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) [10, 166] ou encore le chloroforme [167].

Dans le cadre de ce projet, le recours à ce type de solvants est totalement exclu en raison des applications envisagées (domaines biomédical et alimentaire). De plus, l'idée même d'utilisation de cette catégorie de solvants est contradictoire avec le concept de biocompatibilité, biodégradabilité et non toxicité du chitosane. Ainsi, ce projet s'inscrit dans une vision écoresponsable, basée sur des technologies propres, écologiques et respectueuses de

l'environnement et de la santé publique. En outre, la valorisation des sous-produits de la pêche (dont le chitosane) a pour objectif de réduire notre emprunte carbone sur l'environnement et non l'inverse. Pour ces raisons et pour parer à la mauvaise filabilité du chitosane, nous avons choisi de le mélanger à un polymère non ionique, à structure linéaire et flexible, hydrosoluble et biocompatible: l'oxyde de polyéthylène ou le PEO. Le PEO favorise l'enchevêtrement en entourant et en s'enroulant autour des chaînes du chitosane. Ainsi, le PEO facilite l'électrofilage du chitosane, tout en permettant d'utiliser des solutions aqueuses d'acide acétique.

L'électrofilage coaxial a également été employé pour préparer des nanofibres à base de CS et PEO à structure cœur/enveloppe [11, 168, 169]. Par la suite, certains auteurs ont réussi à obtenir des nanofibres à base de chitosane pur en lavant le PEO dans de l'eau post-électrofilage [168]. Des systèmes nanofibres binaires et ternaires à base de chitosane et d'autres polymères naturels ou synthétiques ont été élaborés. Des échafaudages nanofibreux à base de chitosane, collagène, polycaprolactone (PCL) ont été préparés à différentes proportions pour des greffes de tissus [10]. En plus d'être biocompatibles, les échafaudages ternaires ont montré de meilleures propriétés mécaniques. Des biopolymères comme le collagène, l'acide hyaluronique, l'amidon, la cellulose, l'alginate ont été électrofilés avec le chitosane [170-173]. Plus tard, des nanofibres composites à base de chitosane, cellulose et hydroxyapatite ont été obtenues avec succès [174]. Ces matériaux se sont révélés plutôt efficaces comme échafaudages pour la croissance et la minéralisation de cellules d'ostéoblastes en ingénierie tissulaire. Toujours dans l'optique d'améliorer les propriétés mécaniques des nanofibres tout en exploitant leurs propriétés régénératrices, hémostatiques, antibactériennes et biocompatibles, des électrofilats combinant chitosane et autres polymères naturels et/ou synthétiques additionnés de charges inorganiques ont été préparées. Les matrices polymères étaient constituées par les mélanges chitosane/alcool polyvinylique/cellulose [175], chitosane/lysozyme/alcool polyvinylique [176], acide polylactique/chitosane/collagène [116], le tout renforcé par de l'hydroxyapatite, des nanocristaux de cellulose, des nanoparticules d'or ou d'argent ou encore des nanotubes de carbone.

2.4.3 Paramètres influençant le procédé d'électrofilage

Les paramètres contrôlant le procédé d'électrofilage sont de deux types : (1) les paramètres intrinsèques de la solution polymère, à savoir la concentration, la viscosité, la conductivité électrique, la tension de surface, le poids moléculaire, la structure du polymère (linéaire ou

branché), le DDA (dans le cas du chitosane) et la nature du solvant; (2) les conditions d'électrofilage telles que le débit d'alimentation, le voltage, la distance aiguille-collecteur, la température et le degré d'humidité [177]. Le procédé d'électrofilage est un procédé multifactoriel et une variation d'un ou plusieurs de ces paramètres peut modifier la morphologie des nanostructures obtenues. Ainsi, en jouant sur les paramètres influençant le procédé, on peut moduler le diamètre/taille des nanostructures électrofilées en passant des nanobilles aux nanofibres et vice-versa [178].

2.5 Propriétés antimicrobiennes des nanofibres de chitosane

Les propriétés antibactériennes des solutions et des films de chitosane ont fait l'objet de plusieurs études. En revanche, seules quelques études ont investigué les propriétés antimicrobiennes des nanofibres de chitosane et les informations concernant leur mécanisme d'action sont d'autant plus rares sinon totalement absentes. Il est attendu qu'une plus grande surface spécifique favorise les interactions chitosane–bactérie par le biais des fonctions $-NH_3^+$ qui deviennent plus disponibles et exposées à la surface des nanofibres. Ceci devrait donc renforcer et élargir le spectre d'action antibactérien du chitosane. Qi et son équipe [179] ont rapporté une activité antibactérienne des nanoparticules de chitosane contre *E. coli*, *S.* Typhimurium et *S. aureus* accrue et bien plus élevée en comparaison avec les solutions et les films de chitosane. Les auteurs ont suggéré qu'une plus grande surface offrait un meilleur contact avec la membrane des bactéries.

2.6 Applications des nanofibres de chitosane

Les nanofibres obtenues avec le procédé d'électrofilage ont des caractéristiques remarquablement intéressantes (grande porosité, surface spécifique élevée, diamètre très fin) qui en font d'excellents candidats pour différentes applications dans des domaines variés allant du domaine de la santé et du biomédical au traitement des eaux, en passant par la cosmétologie et le pharmaceutique, entre autres. Le Tableau 2.2 résume quelques-unes des applications les plus courantes mais non-exhaustives des nanofibres à base de chitosane. Mentionnons également la compagnie américaine Tricol Biomedical Inc. qui commercialise des pansements, bandages, bandelettes et autres patches nasaux, dentaires ou encore pour la dialyse. Ces produits à base de chitosane sont commercialisés sous les noms de HemCon® Nasal Plug, HemCon® Dental Dressing Pro, ChitoGauze® Pro, ChitoDot®, etc.

Tableau 2.2: Les principales applications des nanofibres de chitosane.

Domaine	Application	Références
Biomédical	Développement de matériaux fonctionnels nanostructurés. Organes artificiels, greffes	[113] [135]
Santé	Pansement pour plaies opératoires. Exploitation propriétés antibactériennes (*E. coli et S. aureus*) et antifongiques.	[58]
	Membranes non tissées de nanofibres de chitosane pour pansement et cicatrisation des plaies.	[166]
	Fibres de chitosane/collagène pour la cicatrisation des plaies	[20]
	Propriétés antitumorales des nanofibres de chitosane quaternisé	[64]
Ingénierie tissulaire	Membranes support pour la culture d'ostéoblastes et chondrocytes humains. Compatibilité cellulaire.	[126]
	Peaux artificielles, membranes ultra-poreuses de nanofibres de chitosane.	[180] [181]
	Support pour la culture de kératinocytes et fibroblastes humains (cytocompatibilité).	[182]
	Régénération des tissus cartilagineux (Support : nanofibres de CS/PEO).	[183] [184]
	Régénération du tissu osseux, membranes de CS et CS/PVA.	[185] [186] [153]
	Régénération du tissu hépatique, culture de cellules hépatocytes.	[187]
Pharmaceutique	Encapsulation et délivrance de médicaments.	[114] [115]
Biosenseurs	Développement de membranes poreuses support pour l'immobilisation d'enzymes (lipases)	[188] [115]
Technologie de filtration	Complexation, chélation des métaux lourds (Cu et Pb) et des microbes	[106] [6]
Divers	Domaine pharmaceutique et alimentaire : encapsulation de molécules actives lipophiles.	[13] [10]

2.7 Synthèse de la revue de littérature et frontière des connaissances

Le chitosane est un matériau à haute valeur ajoutée et offre une grande variété de propriétés très recherchées dont son origine naturelle, sa biodégradabilité, biocompatibilité et surtout son activité bactéricide. Ces propriétés justifient son utilisation dans des domaines tels que la médecine chirurgicale, le pharmaceutique, la santé, la cosmétologie, l'agroalimentaire et le traitement des eaux. L'électrofilage du chitosane dans le but d'obtenir des nanofibres offre des avantages considérables. En effet, les nanofibres de chitosane (CNFs) possèdent des propriétés remarquables et recherchées dans différentes applications commerciales. Outre leur diamètre ultra-petit (quelques nanomètres), leur très grande surface spécifique (~ 100 m^2/g) et leur grande porosité (> 80 %), les CNFs disposent également de propriétés antibactériennes accrues dues à une meilleure disponibilité de leurs groupements fonctionnels $–NH_3^+$.

Plusieurs études ont démontré l'efficacité antimicrobienne du chitosane sous forme de solutions et de films. Cependant, peu ont examiné les propriétés antibactériennes des nanofibres de chitosane (CNFs). Dans leur article de revue, Martínez-Camacho *et al* [26]. soulignent qu'une étude plus approfondie serait utile pour déterminer si les CNFs présentent le même mécanisme présumé qu'en solution, puisque ce dernier pourrait être affecté par la structure conformationnelle que ces nanomatériaux peuvent adopter [24]. Le mécanisme d'action par lequel le chitosane, en solution, est capable d'inhiber ou de tuer les bactéries, est un phénomène complexe qui n'a pas encore été entièrement expliqué. De plus, aucune information n'est disponible concernant le mécanisme sous-jacent à l'activité antimicrobienne des CNFs. À notre connaissance, aucune étude n'a rapporté le mode d'action des CNFs, ni leur effet sur l'intégrité de la membrane bactérienne. Avant d'envisager des applications commerciales, des études cytologiques de l'effet des CNFs sur la perméabilité membranaire sont nécessaires afin de comprendre leur mécanisme d'action et éviter l'apparition de phénomènes de résistance au chitosane.

Par ailleurs, la quasi-totalité des études disponibles sur le chitosane se sont focalisées sur ses propriétés antimicrobiennes *in vitro* et pratiquement aucune ne s'est penchée sur l'activité antibactérienne des CNFs *in situ* dans des conditions réelles, *i.e*. sur des aliments. S'il est vrai que les expériences *in vitro* fournissent des informations pertinentes quant aux propriétés antibactériennes du chitosane et à son mécanisme d'action, il n'empêche que les microorganismes se comportent différemment lorsqu'ils sont dans un environnement naturel. Les

conditions de croissance incluant la température, le pH, l'activité de l'eau (a_w), la disponibilité des nutriments, la compétition avec d'autres microorganismes ne sont plus des conditions optimales mais réelles, ce qui affecte significativement leur développement. Cette étude est d'une grande importance pour l'utilisation potentielle des CNFs dans différents domaines dont l'industrie alimentaire et le biomédical. Les CNFs étant en contact direct avec soit les aliments (dans le cas de l'emballage) ou la peau (dans le cas des pansements), la compréhension de leur mécanisme d'action devient alors un élément essentiel dans la lutte contre l'altération microbiologique des aliments et les infections de la peau.

CHAPITRE 3 OBJECTIFS DE RECHERCHE, COHÉRENCE DES ARTICLES ET ORGANISATION DE LA THÈSE

3.1 Objectifs de recherche

Sur la base de la revue de littérature présentée dans le Chapitre 2, les études sur les propriétés antibactériennes du chitosane ont donné des résultats contradictoires. De plus, très peu d'informations sont disponibles quant à l'efficacité bactéricide des nanofibres de chitosane (CNFs). Pourtant, la compréhension du mode d'action *via* lequel les CNFs agissent sur les bactéries est une étape essentielle pour maximiser leur spectre d'action antimicrobien avant toute étape de développement de matériaux actifs à base de CNFs. Par conséquent, l'objectif principal de ce travail est le suivant:

" Élaborer de nouveaux matériaux antibactériens et biodégradables à base de chitosane par le procédé d'électrofilage "

Les objectifs spécifiques de la présente étude sont les suivants:

1) Élucider le mécanisme d'action des nanofibres de chitosane.
2) Examiner l'action des nanofibres de chitosane sur la membrane bactérienne.
3) Évaluer l'efficacité antibactérienne des nanofibres de chitosane sur des aliments réels.

3.2 Présentation des articles et cohérence avec les objectifs

Les chapitres suivants comprennent les trois articles qui eux-mêmes contiennent les principales contributions scientifiques et les principaux résultats de ce travail. Cette partie représente donc l'élément central de cette thèse, présenté sous forme de trois articles scientifiques.

Le chapitre 4 présente les résultats du premier article intitulé " *Mechanism of action of chitosan-based nanofibers against meat spoilage and pathogenic bacteria* ", publié le 06 avril 2017 dans le journal *Molecules,* Special Issue: *Antibacterial Materials and Coatings* (vol. 22, issue 4, 1-17, 2017). Dans cet article, nous examinons le mécanisme d'action des CNFs contre les bactéries *Escherichia coli* et *Listeria innocua* (souches non pathogène) et les bactéries *Staphylococcus aureus* et *Salmonella* Typhimurium (souches pathogènes), dans des conditions standardisées

proches des systèmes alimentaires réels. La sensibilité/résistance des bactéries est également examinée en termes de type de Gram, densité de charge, hydrophilie et pathogénicité. Un mécanisme d'action plausible ainsi qu'une explication de la sensibilité/résistance des souches bactériennes aux CNFs sont postulés. L'élucidation du mécanisme d'action des CNFs, selon lequel l'activité bactéricide est due aux fonctions amines protonées est une première. Les résultats obtenus en termes d'activité antibactérienne des CNFs sont prometteurs pour l'utilisation concrète en tant que matériaux antibactériens dans le domaine de l'emballage alimentaire actif ou autres applications où la contamination bactérienne est préjudiciable à la santé du consommateur.

Le chapitre 5 comprend le deuxième article intitulé "*Antibacterial electrospun chitosan-based nanofibers: a bacterial membrane perforator*", publié en ligne le 16 janvier 2017 dans le journal *Food Science & Nutrition* (vol. 5, issue 4, 865-874, 2017). Dans cette étude, l'effet des CNFs sur l'intégrité de la membrane plasmique des bactéries *E. coli* et *S.* Typhimurium est investigué. Les résultats démontrent que l'activité bactéricide des CNFs implique la perméabilisation et la perforation de la membrane, puisque du contenu intracellulaire incluant protéines et ADN a été relargué et détecté dans le milieu extracellulaire et que les observations microscopiques ont permis de mettre en évidence la formation de pores au niveau de la membrane. Une fois le mode d'action exacte des CNFs élucidé, cette étude a permis d'aller plus loin dans le mécanisme d'action. Pour la première fois, les résultats ont permis de mettre en évidence l'effet membranolytique (lyse de la membrane) et perforateur des CNFs, au-delà de leur effet de perturbation/déstabilisation de la paroi.

Le chapitre 6 présente les résultats du troisième article "*Chitosan-based nanofibers as bioactive meat packaging materials*". Cet article a été soumis le 28 mai 2017 au journal *Packaging Technology and Science*. Dans cette étude, est examinée l'efficacité *in vitro* et *in situ* d'un emballage actif à base de CNFs (obtenu par électrofilage direct sur la face interne du multicouche) dans le maintien et l'amélioration de la qualité de la viande rouge fraîche. Cette étude est la première à investiguer l'efficacité bactéricide des CNFs lorsque celles-ci font partie intégrante d'un emballage alimentaire multicouches, et ce dans des conditions réelles comme la lutte contre la contamination de la viande. Les résultats obtenus en termes d'efficacité bactéricide des emballages ainsi activés sont prometteurs pour leur utilisation dans le domaine de la protection des aliments et plus particulièrement dans la conservation de la qualité microbiologique et l'allongement (d'une semaine) de la durée de conservation de la viande.

CHAPITRE 4 ARTICLE 1: MECHANISM OF ACTION OF ELECTROSPUN CHITOSAN-BASED NANOFIBERS AGAINST MEAT SPOILAGE AND PATHOGENIC BACTERIA

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(Cet article a été publié en ligne le 06 avril 2017 dans le journal *Molecules*)

4.1 Abstract

This study investigates the antibacterial mechanism of action of electrospun chitosan-based nanofibers (CNFs), against *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Listeria innocua*, bacteria frequently involved in food contamination and spoilage. CNFs were prepared by electrospinning of chitosan and poly(ethylene oxide) (PEO) blends. The in vitro antibacterial activity of CNFs was evaluated and the susceptibility/resistance of the selected bacteria toward CNFs was examined. Strain susceptibility was evaluated in terms of bacterial type, cell surface hydrophobicity, and charge density, as well as pathogenicity. The efficiency of CNFs on the preservation and shelf life extension of fresh red meat was also assessed. Our results demonstrate that the antibacterial action of CNFs depends on the protonation of their amino groups, regardless of bacterial type and their mechanism of action was bactericidal rather than bacteriostatic. Results also indicate that bacterial susceptibility was not Gram-dependent but strain-dependent, with non-virulent bacteria showing higher susceptibility at a reduction rate of 99.9%. The susceptibility order was: *E. coli* > *L. innocua* > *S. aureus* > *S.* Typhimurium. Finally, an extension of one week of the shelf life of fresh meat was successfully achieved. These results are promising and of great utility for the potential use of CNFs as bioactive food packaging materials in the food industry, and more specifically in meat quality preservation.

Keywords: chitosan-based nanofibers; mechanism of action; gram-negative; gram-positive; meat packaging

4.2 Introduction

Chitosan, a versatile biopolymer generally of marine origin and obtained through chemical or enzymatic deacetylation of chitin, exhibits powerful antimicrobial potential against a wide range of bacteria, fungi, yeasts, viruses, toxins, and spores [1–5]. The availability of chitosan, its affordable cost, non-toxicity, biocompatibility, and biodegradability justify its use in sensitive applications in the biomedical and food industries. Considering food poisoning and waste, two major issues in the food industry, mainly due to microbial contamination or simply an expired shelf life of the product, using active packaging to prevent microbial contamination and the spoilage of food products and consequently extend their shelf life is of major interest for both the food industry and consumers [6–8]. When dissolved in weakly acidic solutions, chitosan has a high density of positive charges due to protonation of its amine functions. This unique characteristic gives rise to many interesting properties among which are a hypocholesterolemic effect, plant defense stimulation, gel formation ability, antioxidant, antiproliferative, antifungal, antibacterial, antiviral, and insecticidal activity [9]. Several studies dating from 1980 have demonstrated the antimicrobial properties of chitosan and its derivatives, with the majority focusing on chitosan solutions and films [10–14]. In their review article, Camacho-Martinez et al. [15] highlighted that there are very few published studies on the antimicrobial properties of chitosan nanofibers and that further investigation in this area will be of great utility for potential applications as bioactive nanomaterials. On the other hand, the main drawback of chitosan is its poor processability. According to Matet *et al*. [16], chitosan shows a degradation temperature lower than its melting point, which prevents the production of chitosan casted films on a large scale and their development in several applications. Furthermore, potential applications of chitosan solutions and films are limited due to poor mechanical and barrier properties.

Electrospinning of chitosan in the form of nanofibers is a promising process that has attracted much interest lately and has been the subject of recent studies [17–23]. The high surface area to weight ratio of the nanofiber mats, their biocompatibility, porosity, small diameter-similar to collagen fibers-and their functional properties make them particularly attractive for various applications such as tissue engineering [24], wound dressings [25], controlled drug release and gene delivery [26], water filtration [27], enzyme immobilization [28], as well as biosensors in the scope of diagnosis [24].

Three possible mechanisms of action have been proposed in the literature to explain the bactericidal activity of chitosan solutions [29–33]. (i) The first mechanism is related to the electrostatic attractions between the positive charges carried by chitosan chains and the negative ones present on the bacterial cell wall. Thus, low and medium molecular weight chitosan can damage the cell membrane through disruption and even perforation, causing the leakage of intracellular components and leading to bacterial lysis and consequently cell death; (ii) The second mechanism suggests that high molecular weight chitosan can form a polymer envelope which encloses the bacterial cell, thus preventing cell exchanges and the absorption of nutrients. Some authors also claimed that in the case of *E. coli*, the predominant mechanism was the first, while for *S. aureus* the second mechanism seemed more likely [34]; (iii) According to the third mechanism, the chelating effect of chitosan is involved in its antibacterial activity. Chitosan would capture trace metals and oligoelements which are essential for bacterial growth, leading to subsequent destabilization of their homeostasis. Other possible mechanisms of action have been proposed in the literature but have been considered as low probability and to be a consequence of one of the aforementioned mechanisms.

Even though the antimicrobial properties of chitosan solutions have been widely reported, the antibacterial activity of CNFs has received much less attention and has been investigated only superficially. Moreover, only a few studies have investigated the exact mechanism of action of chitosan solutions [32,35], microspheres [36], and nanocapsules [37], while CNFs' mode of action has not been addressed yet. For example, Raafat et al. [32] have shown that lipoteichoic acid (LTA) present in Gram-positive bacteria could be involved in the first mechanism of action according to which the positive charges carried by chitosan chains can interact with the negative ones present on the bacterial membrane and cause cellular dysfunction. LTA acts therefore as a molecular link between the bacterial membrane and chitosan chains. However, LTA is a component that is present only in the cell wall of Gram-positive bacteria. Nevertheless, Gram-negative bacteria that lack it are also susceptible to the action of chitosan. Hence, the mechanism underlying chitosan's antibacterial activity and the mode of action by which it inhibits or kills bacteria is a complex phenomenon that has not been fully explained and deserves further investigation [15].

There is considerable controversy in the literature regarding the susceptibility/resistance of Gram-positive and Gram-negative bacteria, to determine whether one or the other is more or less

sensitive to the action of chitosan [29,31,34,38–41]. Hence, it has been established that this difference in strain susceptibility is likely due to structural differences in the bacterial membrane of Gram-positive and Gram-negative bacteria. However, little information is available regarding the involvement of bacterial membrane hydrophobicity/hydrophilicity, surface charge density, as well as pathogenicity in the susceptibility or resistance of both bacterial types.

This study is of great importance for the potential use of CNFs in the food packaging industry. For instance, as CNFs would be in direct contact with the packaged food, understanding their mechanism of action becomes a critical element in the fight against food spoilage and poisoning. In the present work, we examine the mechanism of action of CNFs against food spoilage *Escherichia coli* and *Listeria innocua* and pathogenic *Staphylococcus aureus* and *Salmonella* Typhimurium bacteria, under standardized conditions that mimic real food systems. We also investigate the susceptibility of Gram-positive and Gram-negative bacteria in terms of bacterial type, surface charge density, strain hydrophilicity, as well as pathogenicity. A plausible mechanism of action as well as an explanation regarding the susceptibility/resistance of bacterial strains to CNFs is proposed. To our knowledge, this study is the first that deeply investigates the mechanism of action of CNFs and their bactericidal efficiency in real conditions against meat contamination. The obtained results in terms of the antibacterial activity of CNFs are promising for their utilization as part of the active packaging materials in the scope of food protection and more specifically in meat quality preservation and shelf life extension. Another potential application is the direct use of CNFs as antimicrobial wound dressings to prevent skin infections, which has been the subject of another study [25].

4.3 Results and Discussion

In order to maximize the dose-dependent bactericidal effect of CNFs, it was necessary to use the maximum permissible content of chitosan. The 90/10 (w/w) chitosan/poly(ethylene oxide) CS/PEO formulation generated smooth and homogeneous nanofibers. However, the yield was not efficient because of instabilities (jet fragmentation) that took place during the electrospinning process. On the other hand, CS/PEO nanofibers with ratios less than or equal to 70/30 (w/w) showed a weaker antibacterial activity. Therefore, this formulation (CS/PEO 80/20) was a compromise between the 90/10 ratio that showed the highest antibacterial activity but a low yield of electrospun nanofibers, and the 70/30 ratio which exhibited a lower bactericidal effect but a

higher yield. For the aforementioned reasons, the CS/PEO 80/20 formulation was selected for further characterization and analysis.

4.3.1 Morphology of Electrospun Chitosan Nanofibers

Figure 4.1 presents the effect of molecular weight (M_W) and concentration on the morphology of the electrospun CNFs and their related fiber diameter distributions. The results revealed that the polymer concentration is the key parameter predicting the final morphology and controlling either fiber or particle formation, regardless of the CS/PEO ratio. Our results also demonstrated that at low polymer concentrations, the molecular adhesion between chitosan chains was weak, which leads to electrospraying of the solutions and accordingly to bead formation. When the polymer concentration or M_W increased, allowing sufficient chain entanglement to form a stable filament and prevent its fragmentation, uniform and beadless nanofibers were successfully obtained (Figure 4.1), as also found by Pakravan et al. [17]. Indeed, the minimum concentration required for the formation of continuous and defect free nanofibers depends on a certain polymer concentration (or a multiple of it) which is known as the critical concentration of entanglement (C_e) [17]. C_e is significantly affected by M_w and polymer type (neutral vs. charged, i.e., flexible vs. stiff, respectively). Nevertheless, for the particular chitosan grade of 57 kDa M_W and 95% degree of deacetylation (DDA), which is close to the one used in this study (V3-95/50), Ardila et al. [42] reported a C_e value of 2.5% (w/v). Moreover, McKee et al. [43] found that for neutral polymers, beaded nanofibers start to form at C_e, whilst continuous and defect free nanofibers appear between 2 and 2.5 times C_e. These values reach 8 to 10 times C_e in the case of charged polymers such as chitosan. However, due to the difficulty of achieving such concentrations with chitosan solutions, given the high viscosity and stiffness of the system, the addition of PEO was necessary for nanofiber formation by promoting physical interactions and entanglements. Furthermore, it has been suggested that PEO can possibly interact with chitosan via hydrogen bonding [17], leading to a decrease of the electrostatic repulsions, thus decreasing the viscosity of the system while improving its flexibility and favoring fiber formation. Our results also indicated that the average fiber diameter decreased with chitosan content which was explained by an increase in electrical conductivity (data not shown). Hence, solutions with high chitosan content showed higher repulsive forces, leading to greater stretching and elongation, and consequently to nanofibers with smaller diameter and narrower fiber diameter distribution. The M_W also

contributes in reaching the concentration of entanglement (C_e). Indeed, for a given polymer concentration, it is known that low M_W favors bead formation. On the contrary, high M_W (longer polymer chains) enables the chain entanglement required for fiber formation. Nevertheless, a very high M_W chitosan gives rise to highly viscous and stiff systems which can be difficult or even not possible to electrospin. Overall, electrospinning is a multifactorial process and the electrospinnability of chitosan solutions is known to be severely affected by other parameters such as viscoelastic properties and surface tension of the chitosan solutions. Interestingly, the expected and final morphology of an electrohydrodynamically processed solution can be predicted and tuned by playing with the aforementioned processing and solution parameters.

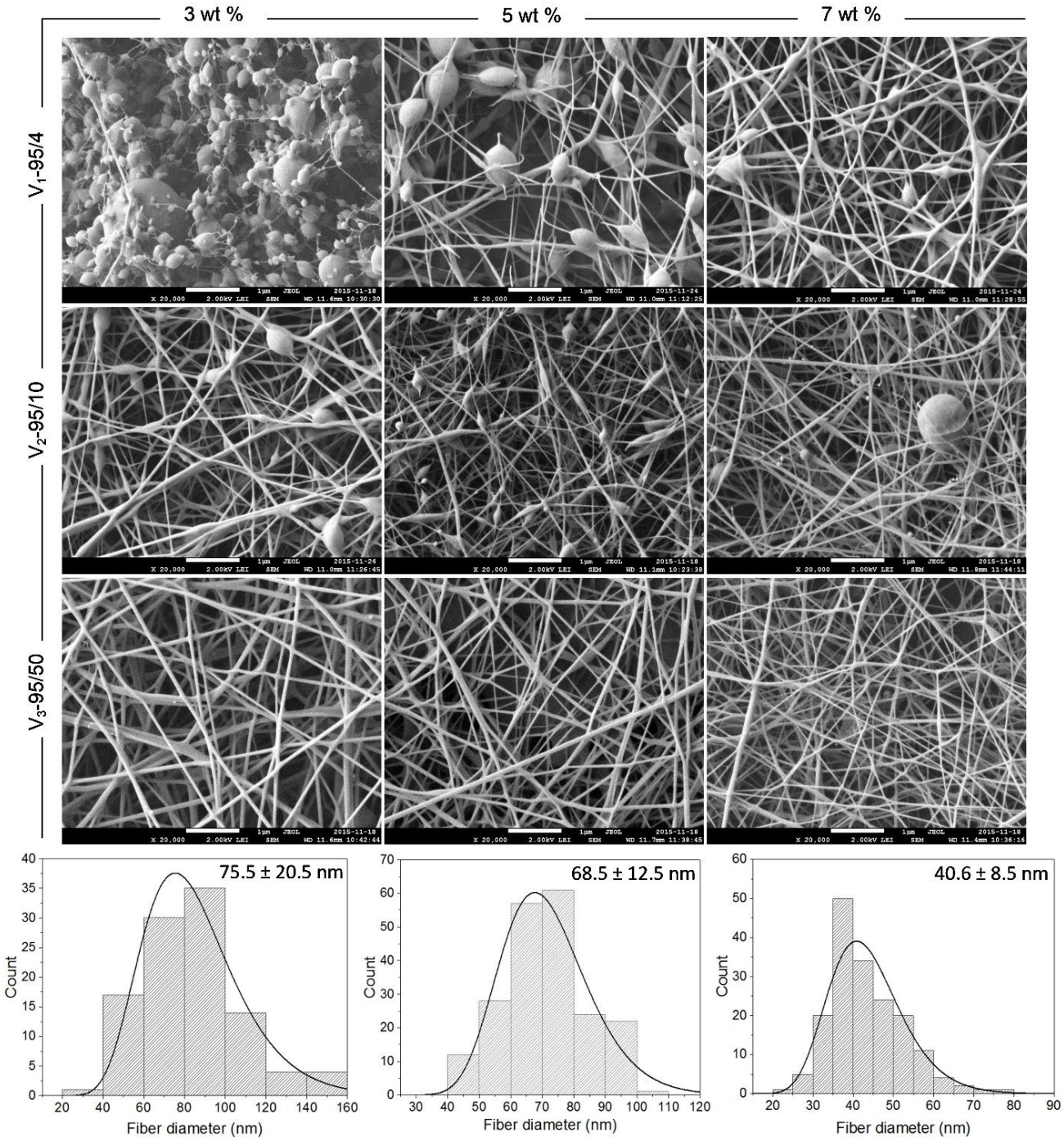


Figure 4.1: Morphology of electrosprayed and electrospun V_1, V_2, and V_3 chitosan nanofibers (CNFs) and fiber diameter distribution of V_3 CNFs. Chitosan's concentrations: 3, 5, and 7 wt % in 50% (v/v) acetic acid (AcOH); Chitosan/poly(ethylene oxide) (CS/PEO) weight ratio: 80/20. All scale bars represent 1 µm.

4.3.2 Mechanism of Action of CNFs-Optical Density (OD_{600})

V_3-95/50 chitosan grade was selected for optical density (OD_{600}) measurements because of its medium M_W, good spinnability, and antibacterial properties, and also because this grade required the lowest concentration for fiber formation (critical entanglement concentration). Figure 4.2a and 4.2b, respectively, show the optical density of *E. coli* and *S.* Typhimurium cultures, in the presence and absence of CNFs. When conditions were optimal, OD_{600} resulted in a typical bacterial growth curve with the different growth phases (black curves). When the cultures were grown in the presence of CNFs, the growth of *E. coli* was completely inhibited while *S.* Typhimurium was severely altered (red curves). When the pH of the suspension was adjusted to neutrality with NaOH in order to deprotonate and inactivate chitosan, no growth recovery was observed. This suggests that the antibacterial effect was irreversible and that CNFs possess a bactericidal effect rather than bacteriostatic, as stated by other authors [32,40]. After CNFs were treated with SDS in order to screen the charges of the NH_3^+ groups, a visible growth with a slight decrease in OD_{600} was recorded (open blue squares), indicating that free amino groups of CNFs were responsible for the antibacterial activity. This slight decrease in optical density may be an artefact due to the lethal effect of sodium dodecyl sulfate (SDS), often used as a lysis solution at higher concentration. The decrease in OD_{600} can also be attributed to chitosan chains that can form a layer which acts as a barrier that prevents cell exchanges. However, even if proven true, it is clear that this mechanism is less intense when compared to the drastic antibacterial effect caused by the positive charges of CNFs (blue squares). When NaCl was also used to screen the positive charges on CNFs (filled blue squares), a similar effect to SDS was observed and the antibacterial activity was severely altered, allowing us to rule out the SDS lysis effect. It is important to mention that at higher salt concentrations (above 5% w/v) than the one used here, NaCl can also cause cell lysis of *E. coli*, as reported by Hrenovic and Ivankovic [44]. Nonetheless, the slight decrease in bacterial growth obtained with the addition of salt is probably due to the fact that some amino groups of CNFs remained protonated, which enabled a slight antibacterial activity. These results strongly indicate that the dominant mechanism of action of CNFs is attributed to their functional protonated amino groups.

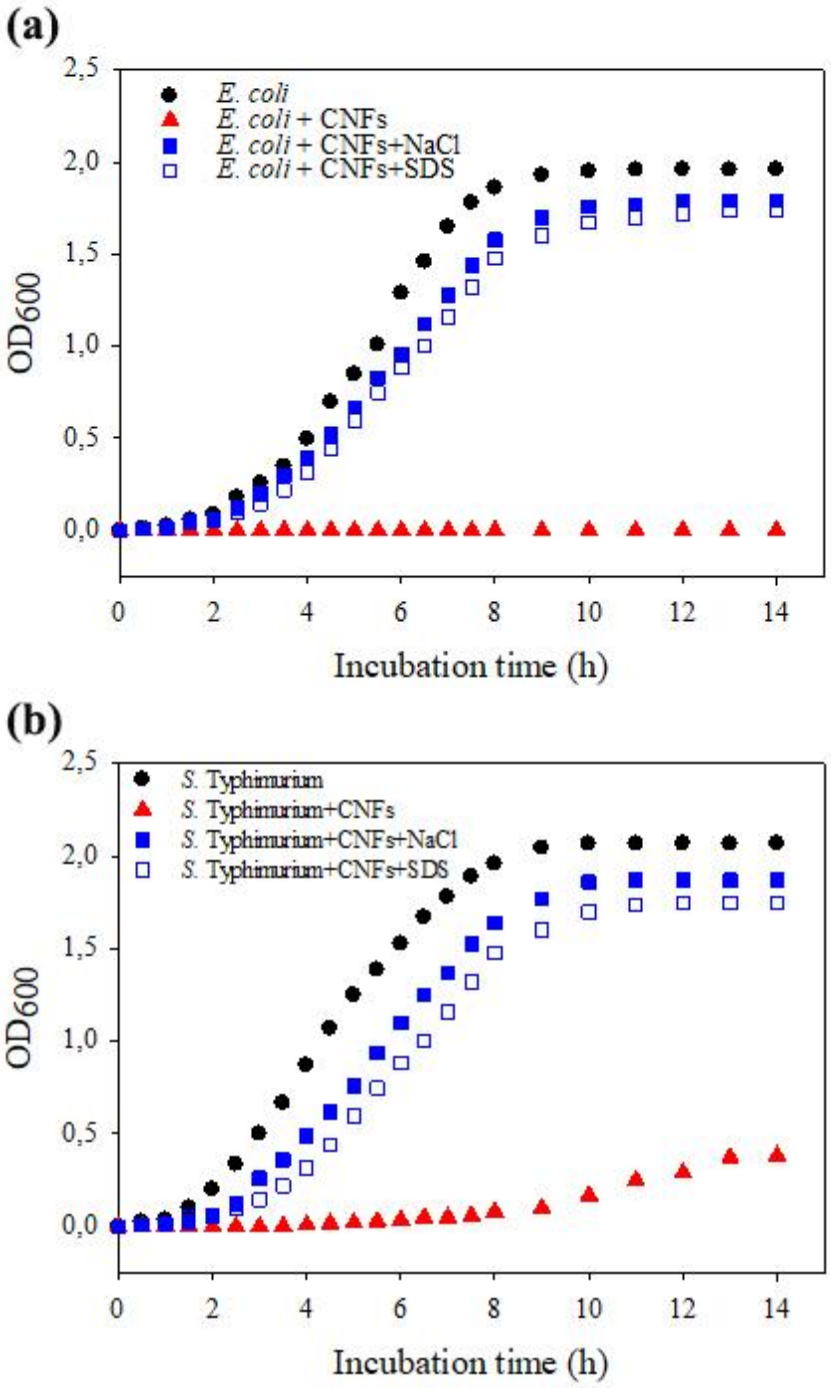


Figure 4.2 : Growth curves of (a): E. coli and (b): S. Typhimurium in the absence (black circles) and in the presence (red triangles) of CNFs (2.5 cm2, V3 95/50, rich Luria-Bertani (LB) medium). Filled and empty blue squares refer to bacterial growth in contact with NaCl and sodium dodecyl-sulfate (SDS)-pretreated CNFs, respectively. The shown data are the mean values of the three replicates method.

4.3.3 MICs and MBCs of Chitosan in Solution State

Table 4.1 reports the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of chitosan (CS) solutions against the tested bacteria, namely two Gram-negative and two Gram-positive model bacteria. MICs and particularly MBCs were necessary to determine the minimum concentration of chitosan that would ensure the antibacterial efficacy of the nanofibers. Our results indicate that CS significantly inhibited (MIC) or killed (MBC) the tested bacteria. However, in the case of pathogenic bacteria such as *S. aureus* and *S.* Typhimurium, the MBC that was necessary to kill 99.9% of these bacteria was 2.5 mg/mL or even higher, a concentration that coincided with the MBC of acetic acid (AcOH). Therefore, it was difficult to separate the contribution of CS from that of AcOH and determine which was responsible for the antibacterial activity. However, experiments (data not shown) conducted in water with the same CS grade revealed that the values of MBCs against *E. coli* were higher in water than in AcOH (2.5 mg/mL against 0.35 mg/mL, respectively), suggesting a synergistic effect between AcOH and chitosan.

Table 4.1: Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of neat AcOH and CS solutions dissolved in aqueous AcOH with concentrations ranging from 0.005 to 5 mg/mL. MICs and MBCs (mg/mL) were determined by the colony forming unit (CFU) method, after 24 h incubation at 37 °C in LB, against the four tested bacteria.

	E. coli		***S.* Typhimurium**		***L. innocua***		***S. aureus***	
Samples	MIC *	MBC *	MIC	MBC	MIC	MBC	MIC	MBC
AcOH	0.50	2.50	2.00	>2.50	0.50	2.50	1.50	2.50
V_1-95/4	0.05	0.15	0.15	≥2.50	0.05	0.15	0.20	0.30
V_2-95/10	0.10	0.30	0.35	≥2.50	0.15	0.30	0.30	0.40
V_3-95/50	0.15	0.35	0.50	≥2.50	0.25	0.40	0.40	≥2.50

***** Results were expressed as mean values of three independent samples and standard deviations represented less than 7% of MIC and MBC absolute values.

4.3.4 Antibacterial Activity of Chitosan Nanofibers

Figure 4.3 shows the antibacterial activity of electrospun chitosan/PEO (80/20) nanofibers (CNFs) against *E. coli*, *S. aureus*, *L. innocua*, and *S.* Typhimurium. Overall, CNFs were very efficient in reducing and stopping bacterial growth at pH 5.8 below chitosan's pKa. To overcome this pH dependence, quaternized chitosan could be used in order to ensure the permanent protonation of cationic sites independently from the pH of the medium [45,46]. A slightly higher

effect against *E. coli* compared with *L. innocua* was observed after 4 h incubation, whilst a reduction of only 2 logs was observed for *S.* Typhimurium, which is not negligible. It is worth mentioning that, surprisingly, there was no effect of one Gram type over the other regarding susceptibility/resistance to CNFs, i.e., Gram-negative bacteria were not more or less susceptible to the action of CNFs than Gram-positive bacteria and vice-versa. More specifically, *E. coli* was significantly more susceptible compared to *S.* Typhimurium and *L. innocua* tended to be slightly more susceptible than *S. aureus* (Figure 4.3). The relative cell surface charge density (RCD) and hydrophilicity appear to be fundamental in understanding the difference in the sensitivities of the bacterial strains. Chung et al. [31] found that these two parameters are correlated with the inhibition efficiency of chitosan solutions (R^2 = 0.942 and 0.824, respectively). Consequently, bacteria that show high RCD and hydrophilicity coefficient (hydrophilicity %) values would have a better affinity, interaction, and adsorption of chitosan chains along their cell wall, leading to a greater inhibition efficiency.

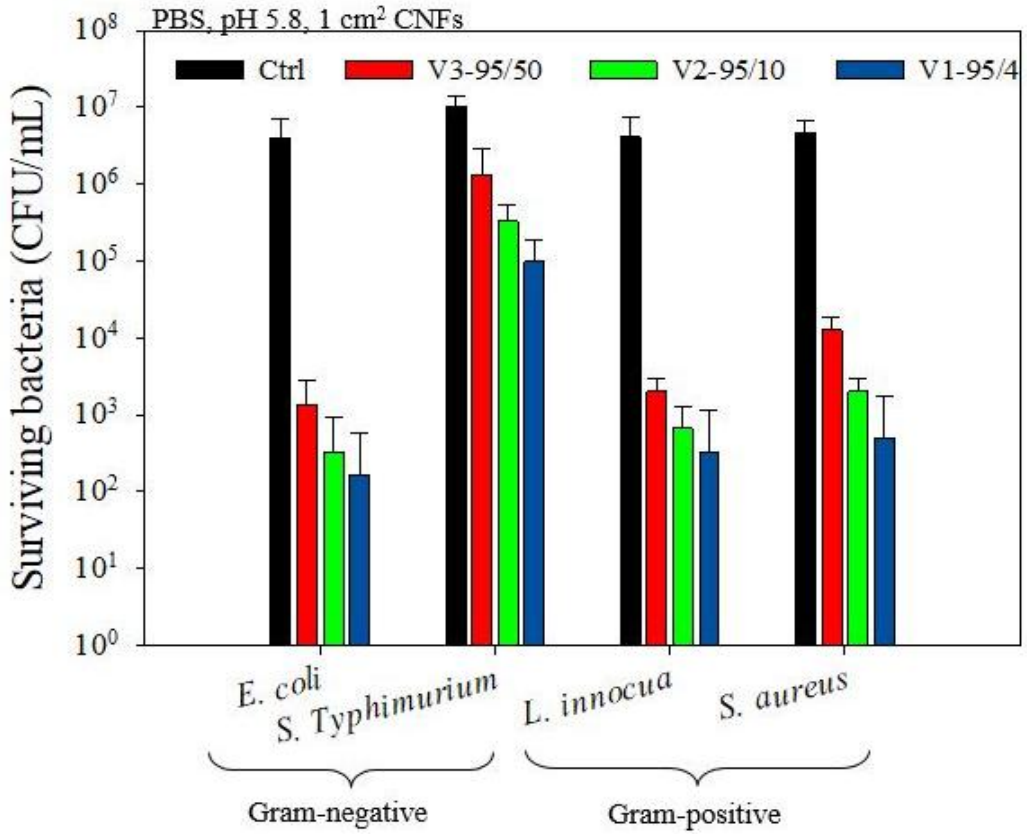


Figure 4.3: Antibacterial activity of electrospun chitosan/PEO (80/20) nanofibers with different M_W against *E. coli*, *S.* Typhimurium, *L. innocua*, and *S. aureus* after 4 h incubation in contact with CNFs.

4.3.5 Kinetics of Bacterial Cell Death and Strain Susceptibility

Figure 4.4 presents the kinetics of bacterial cell death and the sensitivity toward CNFs (1 cm^2 swatches) of Gram-negative (*E. coli* and *S.* Typhimurium) versus Gram-positive (*S. aureus* and *L. innocua*) bacteria at 37 °C in a phosphate buffer saline (PBS, 1 x, pH 5.8). The results show that 99.9% of the Gram-negative *E. coli* was killed after 60 min of exposure, against 180 min for the Gram-positive *L. innocua*, followed by *S. aureus* (240 min), whilst a reduction of only 2 logs was observed for *S.* Typhimurium. The Gram-positive bacteria cell wall is composed of two layers: a thick peptidoglycan layer (murein) overlying the plasma membrane (the target), which consists of a single sheet lipidic bilayer. On the other hand, the cell wall of Gram-negative bacteria is composed of three layers: an outer membrane composed of a phospholipidic bilayer rich in lipopolysaccharide (LPS) and lipoproteins, a thin layer of peptidoglycan, and the inner plasma membrane. The higher hydrophilicity and negative surface charge density (SCD) of Gram-negative bacteria are thought to be mainly due to the presence of LPS [47]. Consequently, the LPS is expected to confer Gram-negative bacteria with a greater affinity to chitosan. In our study, where all the antibacterial tests were conducted in the same in vitro conditions, it was expected that Gram-negative bacteria would be more sensitive to CNFs, independently from M_W, but this assumption did not apply to all Gram-negative bacteria, as observed in Figure 4.4. These results indicate that the antibacterial effect of CNFs is strain dependant rather than Gram dependant, and the strain sensitivity order can be listed as follows: *E. coli* > *L. innocua* > *S. aureus* > *S.* Typhimurium (Figure 4.4). Besides Gram type, other factors such as chitosan-bacterium interaction as well as strain pathogenicity must be taken into account.

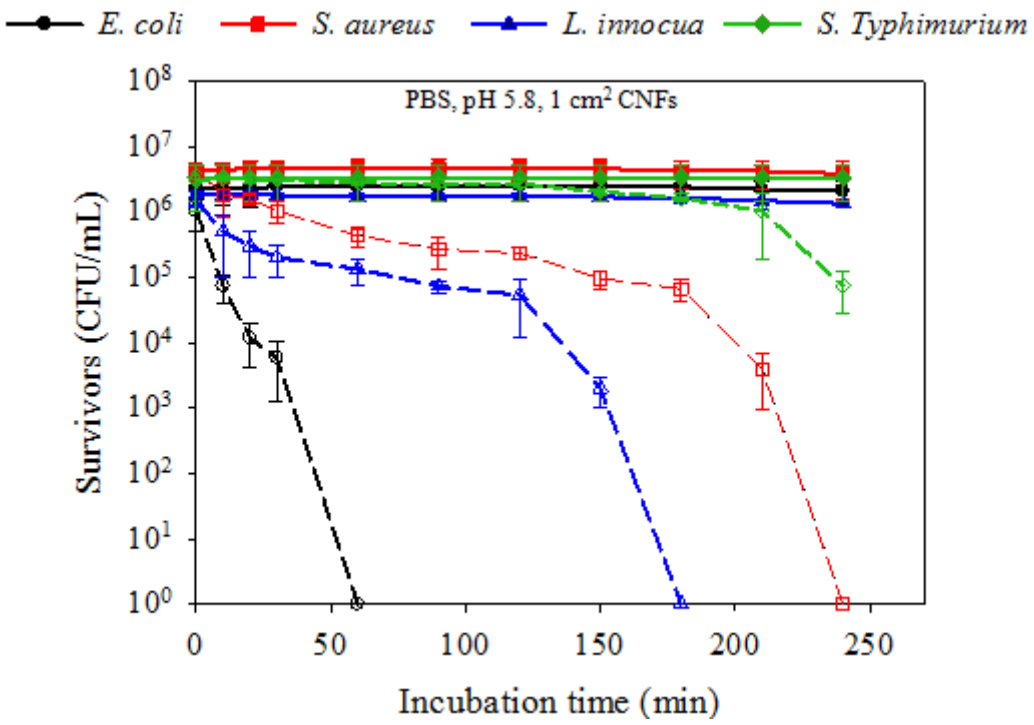


Figure 4.4**:** Kinetics of bacterial cell death induced by CNF (V_3 95/50) on Gram-negative (*E. coli* and *S.* Typhimurium) versus Gram-positive (*S. aureus* and *L. innocua*) bacteria in PBS (1 x, pH 5.8) at 37 °C. Filled symbols refer to controls of bacterial suspension without treatment and empty symbols refer to the same samples after contact with CNFs.

4.3.6 Analysis of Cell Surface Hydrophobicity

Cell surface hydrophobicity and negative surface charge density appear to be fundamental in order to understand the sensitivity difference of the bacterial strains. Figure 4.5 shows the estimation of cell hydrophobicity measured by the bacterial adhesion to a hydrocarbon (BATH) method. The general tendency was that Gram-negative bacteria present a higher hydrophilicity (lower hydrophobicity) than Gram-positive ones. These results are in agreement with those of Chung et al. [31] who found that cell hydrophilicity and SCD are correlated with chitosan's inhibition efficiency. The authors suggested that higher hydrophilicity and negative charge density of the cell surface of Gram-negative bacteria make them more sensitive to the action of chitosan solutions. Recently, some authors have investigated the possible involvement of the LPS in the mode of action of a synthetic aminopeptide (AMP) NK-2 against *E. coli* and *Proteus mirabilis* [48]. Since the LPS containing membrane is the first barrier of Gram-negative bacteria, the authors found that the AMP bound to and intercalated into LPS bilayers, and subsequently induced heterogeneous lesions in bacterial membranes, suggesting that the secondary targets of

NK-2 are intracellular structures, such as DNA. The outer membrane of Gram-negative bacteria is mainly rich in lipopolysaccharides containing phosphate and carboxylic groups, giving the surface a high polar character, hydrophilicity, and density of negative charges in comparison with Gram-positive bacteria [47]. It is then expected that species showing high SCD and hydrophilicity values would have a better affinity, interaction, and adsorption of chitosan chains along their cell wall, leading to greater inhibition efficiency. The expected antibacterial activity should therefore be higher for all Gram-negative bacteria. However, that was not the case and strain susceptibility did not coincide with the hydrophilicity order, as shown in Figure 4.5. Another parameter that may be involved is the pathogenicity of the bacteria. Both *E. coli* and *S.* Typhimurium are Gram-negative but *E. coli* is innocuous while *S.* Typhimurium is pathogenic. The same observation was seen for the two Gram-positive *L. innocua* and *S. aureus* that were investigated here; the first is innocuous while the second is pathogenic. This indicates that hydrophilicity and surface charge density may explain the differences in susceptibility as the strains are innocuous. When pathogenicity is involved, bacteria show resistance toward chitosan, in the same way that some bacteria do not have the same response and show resistance to common antibiotic treatments. Currently, no other satisfactory explanation regarding the observed resistance of *S.* Typhimurium can be given. Chitosan might not be internalized in pathogenic bacteria because of recognition and/or degradation mechanisms. Indeed, further investigation of this behaviour is needed.

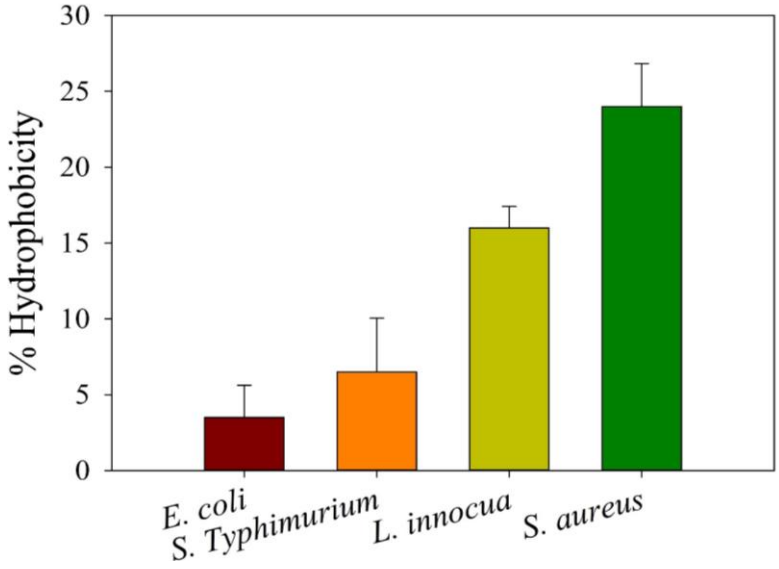


Figure 4.5: Cell surface hydrophobicity of *E. coli*, *S.* Typhimurium, *L. innocua*, and *S. aureus* bacteria, as estimated by the bacterial adhesion to a hydrocarbon (BATH) method.

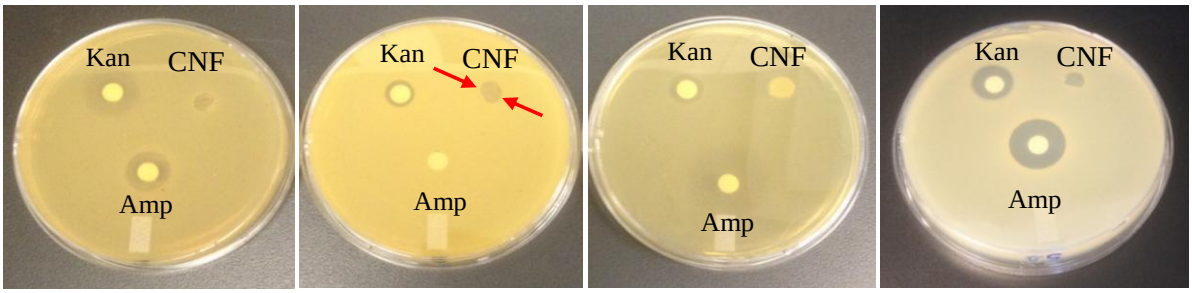
4.3.7 Inhibitory Activity of Chitosan Nanofibers

Figure 4.6 shows the inhibitory activity of CNFs in comparison with two antibiotics, namely kanamycin (Kan) and ampicillin (Amp). CNFs markedly inhibited the growth of the tested bacteria as shown by the inhibition zone inside the nanofiber disks. However, no inhibition area was observed around the disks, in opposition to the two antibiotics. Chitosan did not seem able to diffuse on the agar and form that lysis area around the discs. The high M_W of chitosan in comparison with that of small molecules such as antibiotics may prevent the diffusion of its active sites through the agar. Table 4.2 shows that CNFs nevertheless inhibited the growth of all the tested microorganisms, namely the non-pathogenic bacteria *E. coli* and *L. innocua* and the pathogenic bacteria *S. aureus* and *S.* Typhimurium. Table 4.2 also indicates that the inhibitory effect increased after etching out the PEO from the mats (CNF-PEO sample), thus maximizing the chitosan-bacteria contact. It is important to note that solvent cast chitosan (CS) films (obtained by the evaporation of acetic acid) showed no inhibitory effect on bacterial growth. This is probably due to the greater surface contact area and porosity provided by the nanofibers, which suggests a better bioavailability and adsorption of chitosan functional groups to the bacterial cell membrane. In general, the inhibitory effect of CNFs was nevertheless lower than that of the antibiotics kanamycin and ampicillin. However, the inhibitory power of CNFs against the growth of *S. aureus* was higher than that of Amp, as judged by the higher inhibition zone (6 mm against 0 mm, respectively). It is therefore important to note that *S. aureus* was ampicillin-resistant but chitosan-sensitive. These results suggest that CNFs can be used as potential antibacterial coatings for medical applications, such as wound dressing, implantable medical devices, surgical suture, catheters, contact lenses, and food packaging materials, especially where bacterial development is critical to consumers' health [25,49,50].

Table 4.2: Inhibition zones (mm) of chitosan disks compared to kanamycin and ampicillin antibiotics against *E. coli*, *S. aureus*, *L. innocua*, and *Salmonella* Typhimurium.

Tested Discs	**Inhibition Zone Diameter (mm)**			
	E. coli	*S. aureus*	*L. innocua*	*S.* Typhimurium
CNF	6 ± 0.1	6 ± 0.1	6 ± 0.1	6 ± 0.1
CNF-PEO*	9 ± 0.3	7 ± 0.1	8 ± 0.1	7 ± 0.2
PEO NF	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
CS film	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0
Kanamycin	22 ± 0.4	9 ± 0.1	10 ± 0.2	16 ± 0.2
Ampicillin	18 ± 0.3	0 ± 0.0	24 ± 0.5	19 ± 0.3

CNF-PEO*: refers to CNFs after etching out the PEO by washing with water.



E. coli *S. aureus* *L. innocua* *S.* Typhimurium

Figure 4.6: Antibiogram (Inhibition effect) of CNFs compared to kanamycin (Kan) and ampicillin (Amp) antibiotics, against *E. coli*, *S. aureus*, *L. innocua*, and *S.* Typhimurium. The arrows indicate the inhibition zone caused by chitosan.

4.3.8 CNFs as Active Food Packaging Materials against Meat Contamination

The in situ antibacterial potential of CNFs to extend shelf life and prevent meat contamination by *E. coli* was assessed under refrigeration conditions at 4 °C (Table 4.3). The bacterial initial concentration (inoculum) was 2.5×10^3 colony forming units per milliliter (CFU/mL). The results revealed that when contaminated meat was wrapped in a CNF plus a commercial packaging (MBP-CNFs), bacterial viability was reduced by 92%. Knowing that the initial bacterial concentration used to inoculate the meat was 2.5×10^3 CFU/mL, it is evident that bacteria, fed by the nutrients present in the meat, increased in concentration. This concentration increased by one log order of magnitude and reached 2.5×10^4 and 10^4 CFU/mL in negative (MB-Ctrl$^-$) and positive (MBP-Ctrl$^+$) controls, respectively (Table 4.3). This increase in initial bacterial population was moderate in the positive control, when the samples were wrapped with the conventional packaging in comparison with the unpackaged sample (MB-Ctrl$^-$). This effect was attributed to the good barrier properties provided by the commercial meat packaging which prevented the diffusion of gazes such as oxygen and water vapor, two factors that are essential to bacterial growth. Consequently, further alteration of the meat was limited and slightly slowed down. However, this type of passive packaging was unable to eliminate the bacteria initially present in the sample. PEO nanofibers (sample labelled MBP-PEONFs) were also tested and revealed to be ineffective in inhibiting the growth of *E. coli*. In contrast, CNFs, as part of the active food packaging, eradicated more than 90% of bacterial population, which enabled

preservation of the microbiological quality and safety of the meat and prolonged its shelf life by 7 days at 4 °C.

Table 4.3: Antibacterial efficiency of CNFs against meat contamination by *E. coli*, after 7 day storage at 4 °C. Initial bacterial concentration was 2.5×10^3 CFU/mL.

Samples	Ctrl$^-$ (MB)	Ctrl$^+$ (MBP)	MBP-PEONFs*	MBP-CNFs
Surviving bacteria (CFU/mL)	$2.5 \times 10^4 \pm 0.3$	$1.0 \times 10^4 \pm 0.1$	$1.5 \times 10^4 \pm 0.4$	$2.0 \times 10^4 \pm 0.1$
Reduction rate (%)	-	0.0	0.0	92.2

MBP-PEONFs*: Inoculated meat sample packed in neat PEO nanofibers (PEONFs) plus conventional packaging.

4.4 Materials and Methods

Three water-soluble chitosan (CS) grades (Venzym^{TM} grade) with different molecular weights and a narrow M_W distribution-obtained via enzymatic treatment of chitin-were generously donated by Ovensa Inc. (Aurora, ON, Canada). The various grades are listed in Table 4.4, along with the corresponding nomenclature, M_W, and degree of deacetylation (DDA), provided by the supplier. Poly(ethylene oxide) (PEO) with a M_W of 600 kg/mol and glacial acetic acid (AcOH, 99.7%) were also purchased from Fisher Scientific (Ottawa, ON, Canada).

Table 4.4: Nomenclature, degree of deacetylation, and number average molecular weight (M_n) of the chitosan grades used in this study.

Chitosan (Nomenclature)	DDA[c] (%)	M_n (kg/mol)	Company
V_1 LMW[a]	95	4	Ovensa
V_2 LMW	95	10	Ovensa
V_3 MMW[b]	95	50	Ovensa

[a] low molecular weight. [b] medium molecular weight. [c] degree of deacetylation.

4.4.1 Methods

4.4.1.1 Solution Preparation

Chitosan and Poly(ethylene oxide) solutions were individually prepared at concentrations of 7% and 3% (w/v), respectively, in 50% (v/v) acetic acid. Because of its good spinnability, hydrophilic character, and biocompatibility, PEO was used as a co-spinning agent to improve the spinnability of chitosan, as reported in previous studies [17,51,52]. Solutions were stirred using a

magnetic stirrer for 24 h at room temperature to ensure complete dissolution of the polymer chains. To prepare CS/PEO blends, the solutions were mixed overnight at different blending ratios (50/50, 60/40, 70/30, 80/20, and 90/10).

4.4.1.2 Electrospinning

Electrospinning was performed at room temperature according to Pakravan et al. [17] using a home-made horizontal set-up as shown in Figure 4.7. The solutions were poured into a 10 mL syringe connected to an 18 gauge metal needle. The syringe was placed in a programmable pump (Harvard Apparatus, PHD 2000, Saint Laurent, Qc, Canada) to deliver the required CS/PEO polymer solutions. The metallic syringe was connected to a high voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, USA). A metallic plate or mandrel wrapped with aluminum foil was used to collect the nanofibers in both static and rotating conditions, respectively. The electrospinning processing conditions of CS/PEO solutions are listed in Table 4.5.

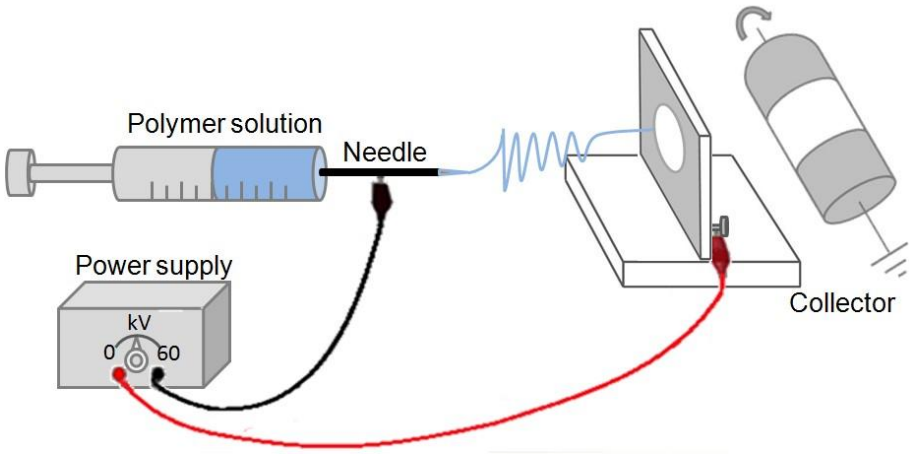


Figure 4.7: Schematic representation of the home-made electrospinning set-up.

Table 4.5: Electrospinning conditions of the CS/PEO and PEO polymer solutions.

Processing Parameters	
Flow rate (mL/h)	0.5
Voltage (kV)	25
Tip-collector distance (cm)	20
Volume (mL)	1–10
Time (h)	2–20
Temperature (°C)	RT * (21)
Relative humidity (%)	7–40

* Room temperature.

4.4.1.3 Scanning Electron Microscopy (SEM)

The morphology of the electrospun chitosan nanofibers was observed with a field emission scanning electron microscope (FESEM Hitachi, JEOL JSM-7600TFE field emission gamma), operated at 2 kV, as described by others [52]. For better conductivity and to reduce electron charging effects, samples were observed as collected on an aluminum foil (without any metallic coating) after 2 h of electrospinning. The spinnability and the presence of beads were also evaluated. The average fiber diameter and fiber diameter distribution were analyzed using Image-Pro Plus® software. Approximately 600 nanofibers randomly chosen from three independent samples (200 nanofibers from each sample) were used for the analysis.

4.4.1.4 Antibacterial Tests

Conditions

Bacterial strains. Escherichia coli (DH5α), *Staphylococcus aureus* (54-73), *Listeria innocua* (ISPQ3284), and *Salmonella enterica* serovar Typhimurium (SL1344), four common foodborne and skin infectious pathogenic bacteria, provided by the laboratory of microbiology, infectiology, and immunology (Université de Montréal, QC, Canada) were used as model bacteria in this study. The strains were kept at 4 °C prior to the testing and then cultured in a broth at 37 °C for 24 h.

Culture media. Luria-Bertani broth (LB) and brain heart infusion (BHI) were used as growing media to start the bacterial cultures. Minimum inhibitory concentrations and minimum bactericidal concentrations of chitosan were determined against the targeted bacteria. LB agar, Muller Hinton agar, and BHI supplemented with agar (15 g/L) were used as solid media for agar plate counting.

Inoculum. Two colonies from the agar plate were re-suspended in 5 mL LB or BHI. The culture was then vortexed and incubated for 24 h at 37 °C under stirring in an orbital incubator shaker (New Brunswick). The final bacterial concentration was approximately 10^9 colony forming units per millilitre (CFU/mL). To achieve 10^6 or 10^3 CFU/mL, bacterial cultures were diluted with a phosphate buffer saline (PBS).

Optical Density (OD_{600})

Optical density at 600 nm wavelength (OD_{600}) using Spectrotonic 200 equipment (Thermo Fisher, Waltham, MA USA) was measured to examine the mechanism of action of chitosan nanofibers (CNFs) and their antibacterial effect on the growth of *E. coli* and *S.* Typhimurium over 24 h at 37 °C. The concept of this method is a measure of turbidity based on the Beer-Lambert law. For this purpose, sodium dodecyl sulfate (SDS 0.01 v/v %), an anionic surfactant, and sodium chloride (NaCl 0.5 M) were used to neutralize CNFs and screen their positive charges. All the experiments were carried out in triplicate and the results were expressed as mean values.

Cell Surface Hydrophobicity

The cell surface hydrophobicity of the tested bacteria was assessed by the bacterial adhesion to a hydrocarbon (BATH) method, as described by Li and McLandsborough [53]. Briefly, a 5 mL broth (LB) was inoculated with 50 µL from an overnight culture. The suspension was then incubated at 37 °C and allowed to grow up to an optical density of 0.5. Thereafter, 4 mL of this suspension was transferred into a 15 mL polypropylene tube (Falcon). A first measurement of optical density OD_{600} was then carried out and recorded as Abs_{t0}. 500 µL of hexane were added to the suspension and the whole mixture was vortexed for one minute and then allowed to rest for one more minute. A second OD_{600} measurement was performed and recorded as Abs_{t1}. Finally, the cell hydrophobicity was calculated according to the following equation.

$$\%\, Hydrophobicity = \frac{Abs_{t_0} - Abs_{t_1}}{Abs_{t_0}} \times 100 \qquad \text{Equation 4.1}$$

Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations of the Chitosan Solutions

The minimum chitosan concentrations necessary to inhibit bacterial growth (MIC) and to kill bacteria (MBC) were firstly determined by the colony-forming unit (CFU) method [54], using chitosan concentrations ranging from 0.005 to 5 mg/mL. Briefly described, the appropriate volume of inoculum (*E. coli*, *S.* Typhimurium, *L. innocua*, and *S. aureus*) was added to reach a bacterial concentration of 10^6 CFU/mL. After 24 h incubation at 37 °C, a 10 µL droplet from

each sample was deposited on top of the LB agar. Finally, the plates were incubated at 37 °C overnight (18 h) for further counting. The MIC and MBC of neat acetic acid solutions were also evaluated. All tests were performed in triplicate and the results expressed as mean values.

In Vitro Antibacterial Efficiency of CNFs

The antibacterial activities of various M_W CS/PEO nanofibers were evaluated against *E. coli*, *S.* Typhimurium, *L. innocua*, and *S. aureus* following the American Society for Testing and Materials (ASTM) standard for antimicrobial agents [54]. Bacteria were grown in LB and BHI broth for 24 h at 37 °C. After incubation, the initial concentration of the bacterial culture was brought from 10^9 to 10^6 CFU/mL by diluting the overnight culture with PBS (1 ×, pH 5.8). The nanofibers (1 cm^2) were then placed into 5 mL of previously prepared bacterial suspension. A negative control of untreated bacteria suspended in PBS was also prepared in the same conditions. Hydrochloric acid (HCl 1 M) was used to adjust the pH of the samples. All the tubes were placed at 37 °C (optimal temperature for bacterial growth) for 4 h incubation in an orbital shaker. Agar plates were inoculated from each tube and then incubated at 37 °C overnight (18 h) for further numeration of survivors. All experiments were carried out in triplicate and the results were expressed as the mean values of three independent samples.

Inhibitory Activity of Chitosan Nanofibers

The inhibitory activity of electrospun CS/PEO nanofibers (CNFs) was evaluated by the inhibition zone diameter (IZD) or agar diffusion method (antibiogram) against the selected model bacteria, by using the slightly modified standard (Clinical and Laboratory Standard Institute—CLSI M02-A12) [55]. The IZDs of CNFs were also compared with those of two standard reference antibiotics, kanamycin and ampicillin (Kan and Amp, 3 µL and 5 µL, respectively). Neat chitosan nanofibers (obtained subsequently to the PEO washing of the mats), PEO nanofibers, and chitosan films (CS films, prepared by solvent evaporation of AcOH) were also analysed. One (1) mL overnight culture of the tested bacteria (10^6 CFU/mL) was spread across the surface of a Muller Hinton agar (MHA) with pH adjusted to 5.8 with 1 M NaOH. Six (6) mm discs of Kan and Amp antibiotics and chitosan nanofibers and films were subsequently deposited on the surface of the agar plate. The plates were then incubated overnight (18 h) at 37 °C.

CNFs as Active Packaging Materials against Meat Contamination

Meat preservation tests were performed in order to assess the antibacterial activity of CNFs under real conditions. Briefly, 10 g of fresh meat cubes were cut under aseptic conditions. Samples were inoculated by a 30 s immersion in a bacterial suspension of *E. coli* (10^3 CFU/mL) and were wrapped in CNF mats, immediately after drying. In order to compensate for the poor mechanical and barrier properties of CNFs, inoculated meat samples were also packaged in a conventional co-extruded multilayer food packaging (sample labelled MBP-CNFs; M for meat, B for bacteria, and P for packaging). The commercial multilayer packaging was composed of poly(ethylene terephthalate) (PET) and ethylene vinyl alcohol (EVOH), provided by ProAmpac (Terrebonne, QC, Canada). Samples were then sealed under vacuum and finally stored at 4 °C for further analysis. Negative MB–Ctrl$^-$ and positive MBP–Ctrl$^+$ controls of inoculated meat, wrapped with and without conventional packaging but without CNFs, were also prepared under the same conditions. The surviving bacteria were collected by grinding the meat cubes with a laboratory tissue grinder to separate bacteria from the surface of the meat tissues. After serial dilution, samples were spread on top of LB agar plates and incubated at 37 °C for 24 h for further counting of survivors. Finally, the reduction rate of the bacteria population was calculated according to the following equation [56]:

$$R\ (\%) = \frac{N_0 - N}{N_0} \times 100 \qquad \text{Equation 4.2}$$

where N_0 and N are the numbers of colony forming units (CFU, before and after CNF treatment, respectively. The number of colony forming unit was determined as follows:

$$\mathrm{CFU/mL} = \frac{\text{number of colonies}}{\text{dilution factor} \times \text{volume (mL)}} \qquad \text{Equation 4.3}$$

4.5 Conclusions

This study is the first that investigates the mechanism of action of CNFs against Gram-negative and Gram-positive bacteria; including the strain susceptibility/resistance toward CNFs. Our in vitro results demonstrate that the predominant mechanism of action of CNFs is attributed to their functional protonated amino groups, regardless of bacterial type. Our results strongly indicate

that susceptibility was not Gram-dependent, as stated in the literature, but strain-dependent. In addition, in contrast to what is stated in the literature, our findings show that chitosan's irreversible antibacterial effect is bactericidal rather than bacteriostatic. The CNFs studied here were very efficient in reducing and stopping microorganism growth at pH 5.8 below chitosan's pKa. To overcome this pH dependence, it is possible to restrict the use of CNFs to foods having an intrinsic weakly acidic pH such as milk, yogurts, cheeses, fish, and meat, whose pH acidifies as lactic acid is released during storage. The in situ antibacterial tests showed the potential of CNFs as bioactive nanomaterial barriers to meat contamination and showed their ability to maintain safety and extend the shelf life of fresh red meat by one week. However, another issue that may limit the use of CNFs as active food packaging is that their effectiveness is strictly conditional on contact with the packaged food, narrowing the potential applications to vacuum packaging of food products such as fresh meat, sausage, charcuteries, chicken skewers, ribs, smoked meat and salmon, fish, etc. To overcome this issue, it may be envisaged to combine the antibacterial action of CNFs with that of certain essential oils for a synergistic effect. Overall, the extension of the expiration date of unprocessed and preservative-free foods could facilitate the logistics of the whole production chain including distribution and storage, while ensuring the quality and safety of the packaged product for consumers.

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Author Contributions: Mounia Arkoun conceived the study, performed the experiments, collected test data, analyzed and interpreted the results, and drafted the manuscript. France Daigle, Marie-Claude Heuzey, and Abdellah Ajji conceived the study, interpreted the results, and reviewed and edited the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Some samples of the compounds (chitosan nanofibers) could be available from the authors upon request.

CHAPITRE 5 ARTICLE 2: ANTIBACTERIAL ELECTROSPUN CHITOSAN-BASED NANOFIBERS: A BACTERIAL MEMBRANE PERFORATOR

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5.1 Abstract

This study investigates the antibacterial action of chitosan-based nanofibers (CNFs) obtained by the electrospinning process on the permeability of bacterial membranes. The bactericidal efficiency of CNFs was firstly determined against Gram-negative *Escherichia coli* and *Salmonella* Typhimurium and Gram-positive *Staphylococcus aureus* and *Listeria innocua* bacteria as a baseline. The results strongly suggest that CNFs interact with the negatively charged bacterial cell wall causing membrane rupture and inducing leakage of intracellular components among which are proteins and DNA. Results clearly indicate that the release of such components after contact with CNFs is an indication of membrane permeabilization and perforation, as pore formation was observed in transmission electron microscopy (TEM). This work suggests a plausible antibacterial mechanism of action of CNFs and also provides clear evidence in favor of chitosan as a bacterial membrane disruptor and perforator. As a result, CNFs can find promising applications as bioactive food packaging materials capable to extend shelf life of food products while inhibiting the spread of alteration flora and foodborne pathogens.

Keywords: antibacterial; electrospun chitosan-based nanofibers; membrane permeability.

5.2 Introduction

Electrospinning of chitosan, with the aim of producing nanofibers with diameters ranging from few tens of nanometers to micrometers, has been the subject of several recent studies (Desai, Kit, Li, & Zivanovic, 2008; Doğan, Özyıldız, Başal, & Uzel, 2013; Elsabee, Naguib, & Morsi, 2012; Geng, Kwon, & Jang, 2005; Homayoni, Ravandi, & Valizadeh, 2009; Kriegel, Kit, McClements, & Weiss, 2009; Pakravan, Heuzey, & Ajji, 2011; Rieger, Birch, & Schiffman, 2016; Ziani et al., 2011). The resulting chitosan nanofiber (CNF) mats exhibit a remarkably high porosity (in the range of 80 to 90 %) and surface area per unit mass (between 10 and 500 m^2/g) and display good biocompatibility and biofunctionality (Ardila et al., 2016; Greiner & Wendorff, 2007). Therefore, CNFs may have promising applications in biomedical (cell culture, wound healing, tissue engineering) (Ignatova, Manolova, Markova, & Rashkov, 2009), pharmaceutics (controlled drug release, gene therapy) (Jayakumar, Prabaharan, Nair, & Tamura, 2010), water filtration (chelation of metal ions) (Haider & Park, 2009) and food packaging (Martínez- Camacho et al., 2011), among others. However, achieving high yield and quality fiber formation from neat chitosan solutions is a challenging task. This is mainly due to the very rigid structure of chitosan chains, which does not promote entanglements that are required for the formation of the Taylor cone, which in turn generates nanofibers. For example, some authors reported the preparation of neat CNFs using trifluoroacetic acid (TFA) as a solvent or its mixtures with dichloromethane (DCM) (Gu et al., 2013; Lee et al., 2014). However, TFA is highly cytotoxic, corrosive and environmentally harmful, making the use of such materials incompatible with applications as delicate as food packaging. Moreover, electrospinning is a multifactorial process that involves several parameters among which processing conditions such as flow rate, electric field, collecting distance, temperature and humidity, as well as intrinsic solution parameters including conductivity, surface tension and viscoelasticity. Thus, in order to improve the electrospinnability of chitosan, a co-spinning agent at moderate content is often needed and used as a carrier polymer to trigger fiber formation (Moayeri & Ajji, 2015; Rieger et al., 2016).

Studies have demonstrated that chitosan, in the form of solution and films, exhibits efficient antimicrobial activity (Muzzarelli et al., 1988; Papineau, Hoover, Knorr, & Farkas, 1991; Shahidi, Arachchi, & Jeon, 1999; Sudarshan, Hoover, & Knorr, 1992; Young, Köhle, & Kauss, 1982). However, few have examined the antibacterial properties of CNFs. In a review article,

Martínez- Camacho et al. (2011) point out that most reports on the antimicrobial activity of CNFs have used chitosan solutions instead. In most cases, the proposed mechanism for CNFs was indirectly related to the presence and release of protonated amino groups from CNFs mats, which were no longer nanofibers. The authors highlighted that further investigation would be useful in order to determine whether CNFs follow the same presumed mechanism, since it might be affected by the structural conformation these nanomaterials can adopt (Kong et al., 2008). The mechanism of action by which chitosan, in solution state, is able to inhibit or kill bacteria is a complex phenomenon that has not been fully explained either (Hammer et al., 2010; Kong, Chen, Xing, & Park, 2010; Raafat, Von Bargen, Haas, & Sahl, 2008). Moreover, no information is available regarding the mechanism underlying the antimicrobial activity of CNFs. To our knowledge, no study has reported the effect of CNFs on bacterial cell membrane integrity, nor their mode of action. A cytological study of the effect of CNFs on the bacterial membrane permeability is necessary to understand their exact mechanism of action and to avoid the outbreak of potential resistance phenomena. In this study, we investigate the antibacterial mechanism of action of CNFs against four common alteration flora and foodborne pathogens, most frequently incriminated in food spoilage and food poisoning, respectively. All tests were performed under standardized and controlled experimental conditions to facilitate reproducibility and allow comparative studies. A plausible mode of action in which CNFs act as membrane permeability disruptor and even perforator is postulated. In this context, CNFs represent ideal biomaterials that can be used as suitable bactericidal barriers to prevent bacterial infections in several areas, including food packaging and biomedical applications. As part of active food packaging, CNFs can be applied to extend the shelf life of food products and prevent spoilage and foodborne diseases caused by *Escherichia coli*, *Listeria*, *Staphylococcus*, and *Salmonella*.

5.3 Materials and methods

5.3.1 Chemicals and polymers

Water-soluble chitosan (CS), a Venzym^{TM} grade obtained *via* enzymatic treatment of chitin derived from shrimp shells was generously donated by Ovensa (Ontario, Canada). The water-solubility of this CS grade is due to the presence of a low amount of residual acetic acid (AcOH), as confirmed by the supplier. The corresponding degree of deacetylation (DDA) and number

average molecular weight (M_n) are 95% and 50 kDa, respectively, with a narrow molecular weight distribution. Poly(ethylene oxide) (PEO), a co-spinning agent for chitosan, with a molecular weight of 600 kDa, and acetic acid (AcOH, glacial, 99.7%) were purchased from Fisher Scientific (Saint-Laurent, QC, Canada). All materials were of analytical grade and used as received.

5.3.2 Microorganisms, culture media and conditions

Bacterial strains

Escherichia coli (DH5α), *Salmonella* Typhimurium (SL1344), *Staphylococcus aureus* (54-73) and *Listeria innocua* (ISPQ3284) were supplied by the Laboratory of Microbiology, Université de Montréal (Québec, Canada). Cultures were maintained at 4 °C prior to use, then transferred into a culture medium and finally incubated at 37 °C for 24 h in an orbital shaker (New Brunswick) to achieve an initial concentration of 10^9 colony forming unit per milliliter (CFU/ml).

Culture media

Luria-Bertani (LB) broth and brain heart infusion (BHI) were used as growth media to start the bacterial cultures. To reach the required final concentration, cultures were diluted using phosphate buffer saline (PBS, pH 5.8, adjusted with 1 mol/L HCl). LB agar and BHI supplemented with agar (15 g/L) were used as solid media for counting the surviving bacteria.

5.3.3 Preparation of chitosan and PEO stock solutions

Chitosan (CS) and (PEO) stock solutions (7% w/v and 3% w/v, respectively) were individually prepared by dissolving polymer powders in 50% (v/v) AcOH under overnight magnetic stirring. The CS/PEO blends were obtained by magnetic stirring of the two polymer solutions in a proportion of 80/20 (w/w) ratio for 4 h agitation. The advantage of using aqueous acetic acid solutions is their non-toxic and eco-friendly character.

5.3.4 Preparation of chitosan-based nanofibers *via* electrospinning

CS/PEO nanofibers were prepared according to Pakravan et al. (2011) using the electrospinning process. Electrospinning of the blend solution was performed using a horizontal homemade setup containing (1) a high voltage power supply (Gamma High Voltage Research, FL, USA), (2) a programmable pump (Harvard Apparatus, PHD 2000) to deliver the polymer solution at the

required flow rate, (3) a metallic rotating drum wrapped with aluminum foil to collect the nanofibers. A schematic representation of the set up is shown in Figure 5.1. The electrospinning blend solution was poured into a 10 mL syringe with Luer–Lock connection to an 18-gauge blunt tip needle (Cadence Science, USA). The syringe was mounted on the pump with a grip and grounded by use of an alligator clip. The optimal process parameters were flow rate of 0.5 ml/h, voltage of 20 kV and needle tip-to-collector distance of 15 cm. All experiments were conducted at room temperature (22 ± 1ºC), relative humidity of 20% and under atmospheric pressure. The collected nanofibers were dried overnight under a hood to ensure complete evaporation of the solvent.

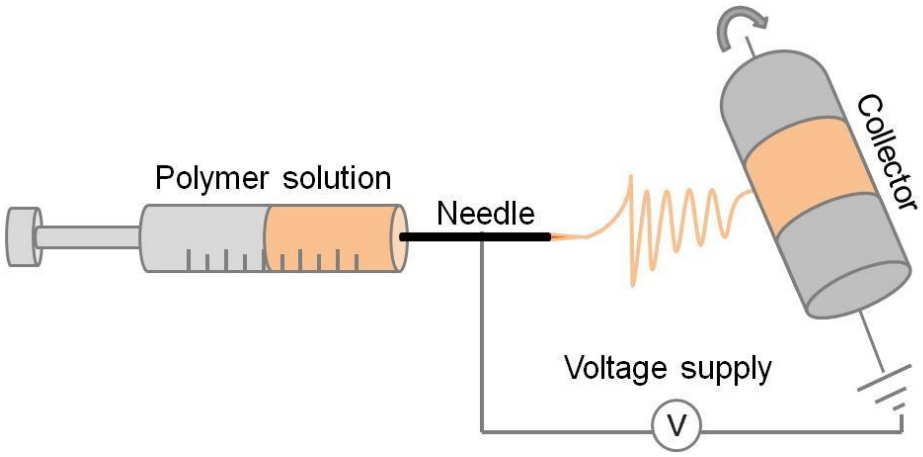

Figure 5.1: Schematic representation of the homemade electrospinning set up.

5.3.5 Scanning electron microscopy (SEM)

The morphology of the electrospun chitosan-based nanofibers (CNFs) was examined according to a slight modified method of Moayeri and Ajji (2015), using a field emission scanning electron microscope (FESEM JEOL JSM-7600TFE), operated at 1.5 kV. Samples were observed as collected on an aluminum foil after 2 hr electrospinning. SEM results revealed that uniform and beadless fibers were obtained in the presence of the co-spinning agent, PEO in this specific case. The average fiber diameter was evaluated using Image-Pro Plus® software. Approximately 600 nanofibers randomly chosen from three independent electrospun mats (200 fibers from each sample) were used for the quantification of fiber morphology parameters.

5.3.6 Antibacterial efficiency of CNFs

The antibacterial activity of electrospun CNFs was evaluated in vitro following the American standard test method (ASTM E2149−13a, 2013). Commonly found bacteria, *E. coli, S. aureus, L. innocua* and *S.* Typhimurium, in food contamination and skin infections were selected for this purpose. Samples of 1 cm^2 and 2.5 cm^2 swatches of CNFs were prepared in aseptic conditions. Bacterial suspensions (10^6 CFU/ml, 5 ml PBS, pH 5.8) were put in contact with CNFs. It is noteworthy that even though the CS grade used in this study was water-soluble, the resulting nanofibers were visually insoluble in aqueous media post-electrospinning due to solvent evaporation during processing. Negative controls of bacteria suspended in PBS without CNFs were also prepared. All tubes were placed at 37°C, optimal temperature for bacterial growth, for 4 hr incubation in an orbital shaker. Serial dilutions were performed and spread on agar plates incubated overnight at 37°C for further counting of survivors. All tests were conducted in triplicate. Finally, the antibacterial efficiency was expressed as a function of the reduction rate (*R*) of the total number of test bacteria. *R* was calculated according to Belalia, Grelier, Benaissa, and Coma (2008) using the following equation:

$$R\ (\%) = \frac{\mathrm{A-B}}{\mathrm{A}} \times 100 \qquad \text{Equation 5.1}$$

where, *A* and *B* are the numbers of surviving bacteria in the controls and test samples, respectively.

5.3.7 Effect of chitosan-based nanofibers on membrane permeability

5.3.7.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE)

The release of intracellular proteins from CNF-treated bacteria was investigated by SDS-PAGE. In this section, *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) were selected in order to appraise the effect of Gram type on the strains' susceptibility/resistance to CNFs. Overnight cultures of *E. coli* and *S. aureus* were resuspended in PBS (~10^8 CFU/ml) and incubated at 37°C in the presence of CNFs. After 0, 1, 2, 3 and 4 hr contact time, 5 ml aliquots were withdrawn and centrifuged at 3,000 *g*/10 min at 4°C. The supernatants were then mixed with trichloroacetic acid (TCA 10:1) and left for precipitation at 4°C overnight. After a series of wash, samples were resuspended in SDS-loading buffer and subjected to SDS-PAGE according to the method of

Laemmli (1970). Positive controls ($Ctrl^+$) of extracted proteins from *E. coli* and *S. aureus* were also prepared by chemical lysis of both bacteria using a lysis solution containing 50 µl of chloroform and 25 µl of SDS (0.5% v/v). For more sensitivity, revelation was performed by using silver nitrate staining of proteins.

5.3.7.2 Agarose gel electrophoresis of released DNA

Because of its importance in fundamental research, its use in the industrial field and its involvement in the agri-food sector, the *E. coli* laboratory strain has been fully sequenced and its genome is currently 100% known. In the following section, *E. coli* (DH5α) bacterium was chosen to study the effect of CNFs on membrane permeability and subsequent DNA leakage.

The leakage of DNA from CNF-treated *E. coli* was investigated by agarose gel electrophoresis as an indication of membrane damage. DNA was extracted from CNF-treated *E. coli* cultures according to the protocol of Green and Sambrook (2012). Briefly, 5 ml aliquots were subjected to centrifugation (6,000 rpm, 10 min at 4°C), filtration (0.22 µm pore size) and overnight precipitation at -20°C in sodium acetate (NaAc 3 mol/L pH 5.2) and ethanol (EtOH 100%, -20°C, 2.5 x volume). Samples were centrifuged (9,000 *g*, 15 min, 4°C) and the resulting pellets were suspended in ethanol (70%, -20°C), centrifuged again, dried under the hood and resuspended in milliQ water. Positive controls of bacterial DNA extracted from *E. coli* after chemical and heat treatment ($CtrlL^+$ and $CtrlH^+$, respectively) were also prepared. An additional step of pH adjustment (pH 7.0) with 1 mol/L NaOH in order to deprotonate the CNFs and break up CS-DNA interactions was necessary. A polymerase chain reaction (PCR) for the *rrnB* gene 16S RNA was performed in order to amplify the released DNA fragments from chitosan-treated cultures. Finally, DNA extracted sequences were loaded on a 2% (w/v) agarose gel and migrated for 20 min at 90 V. DNA quantification was also performed using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific).

5.3.7.3 β-galactosidase assay

In this section, *E. coli* DH5 hxt 55632–*Lac* Z^+, a strain that overexpresses the gene encoding the β-galactosidase (β-gal) activity (without addition of lactose to the medium) was selected to assess the effect of CNFs on membrane permeabilization. To this end, the release of intracellular β-gal was evaluated by enzymatic titration according to Miller (1992). An overnight culture was

diluted in LB and brought to an optical density (OD_{600}) of 0.6, using a spectrophotometer (Spectrotonic 200; ThermoFischer). The suspension was then incubated at 37°C, in the presence (treated samples) and absence (negative control, $Ctrl^-$) of CNFs at different contact times. A positive control ($Ctrl^+$) of lysed cells was prepared by adding 50 µl of chloroform and 25 µl of SDS (0.1% v/v) to the culture. A volume (*v*) of 50 µl of each sample was diluted in 950 µl of neutral buffer (Z buffer, pH 7.0) over an ice bath. Samples were placed for 5 min at 28 °C in a water bath before starting the reaction. To each sample, 200 µl of *o*-nitrophenyl-β-galactoside (ONPG, 4 mg/ml) was added and the reaction was timed. When samples turned yellowish, the reaction was stopped by adding 500 µl of 1mol/L Na_2CO_3 and the time recorded (*t*). Tubes were then centrifuged 2 min at 13,000 *g* to remove cell residues and the optical density of the supernatant was measured at 420 nm and 550 nm (OD_{420} and OD_{550}). Finally, the β-galactosidase activity, expressed in β-gal units or Miller units was calculated using the following equation:

$$\beta - \text{galactosidase units} = \frac{1000 \times (OD_{420} - 1.75 \times OD_{550})}{t \times v \times OD_{600}} \qquad \text{Equation 5.2}$$

5.3.7.4 Transmission electron microscopy (TEM) analysis of bacterial membrane integrity

TEM was performed to investigate the effect of CNFs on cell morphology and membrane integrity. Sample preparation was performed following the guidelines of Tao, Qian, and Xie (2011) and Xing et al. (2009a) with a slight modification. Overnight cultures (10^6 CFU/ml) of the selected bacteria were exposed to CNFs for 10, 20, and 30 min. Cultures were then centrifuged (60,00 *g*/3 min) and the resulting pellets were resuspended in a 2% (v/v) glutaraldehyde solution contained in PBS (pH 7.4) for overnight fixation of the cells at 4°C. A quantity of 10 µl of each sample was deposited on Formvar carbon-coated grids containing one drop of 1% Alcian Blue. Cells were then subjected to 5 min post-fixation with 2% paraformaldehyde in PBS, and grids were stained using a drop of filtered 2% phosphotungstic acid (PTA, pH 7.0) for 30 s. A series of filtration and/or washing treatment was performed after each step to remove excess liquid, fixative and staining. Untreated bacteria samples were also prepared by the same method. Finally, TEM observation was performed using a Philips CM100 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) and digital micrographs were captured using an AMT XR80 CCD digital camera (Advanced Microscopy Techniques, Woburn, MA USA).

5.4 Results and discussion

5.4.1 Morphology of electrospun CNFs

Figure 5.2 shows SEM images of electrospun CNFs from 7% (w/v) CS solution in 50% (v/v) AcOH, and 80/20 wt ratio CS/PEO blend in 50% (v/v) AcOH. As shown in Figure 5.2a, electrospinning neat CS was a difficult task and mostly gave rise to particles of nanometer and micrometer size (electrospraying). Hence, the addition of a cospinning agent such as PEO to facilitate the electrospinnability of CS was unavoidable. When added to CS solution in a moderate proportion (CS/PEO wt ratio: 80/20), PEO could act as a carrier and improve the viscoelastic properties of CS solution as well as chain entanglement and flexibility (Pakravan et al., 2011), two *sine qua non* conditions for fiber formation. Consequently, homogeneous and beadless chitosan-based nanofibers with average fiber diameter of 78 nm ± 22 were successfully obtained.

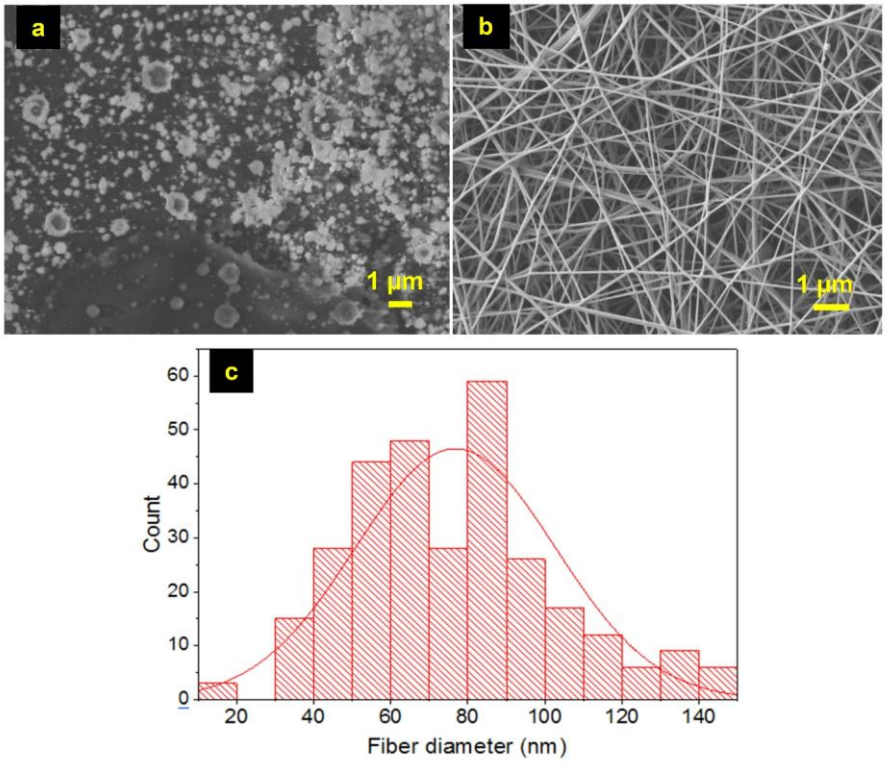

Figure 5.2: SEM micrographs of (a): electrosprayed 7% (w/v) CS in 50% (v/v) AcOH and (b): electrospun 7% (w/v) CS/PEO (80/20) in 50 % (v/v) AcOH at 21°C, 7% relative humidity, (c): Fiber diameter distribution of b. Process parameters: tip-to-collector distance = 15 cm, flow rate = 0.5 ml/hr, voltage = 20 kV. Scale bars represent 1 µm diameter and magnification x6 and x10 for samples 2a and 2b, respectively.

5.4.2 Antibacterial efficiency of CNFs

Table 5.1 and Figure 5.3 display, respectively, the bacterial reduction rate (*R*) and the in vitro antibacterial activity of CNFs, quantitatively assessed by the CFU method against *E. coli*, *S.* Typhimurium, *L. innocua* and *S. aureus*. After 4 hr contact at 37°C in PBS (pH 5.8), CNFs (1 cm^2) showed significant reduction rate ($R > 99\%$) of bacterial growth of *E. coli*, *L. innocua* and *S. aureus* (Table 5.1), versus 96.91% for *S.* Typhimurium. When CS content was increased (2.5 cm^2 instead of 1 cm^2), CNFs were able to completely stop the growth of *E. coli* and *L. innocua*, (100% *R*, Table 5.1), as shown by the arrows (Figure 5.3). However, *S. aureus* and *S.* Typhimurium showed lower susceptibility to the action of CNFs. Nevertheless, a significant dose-dependent decrease of bacterial population (5 logs and 4 logs, respectively) was still observed (Figure 3). Furthermore, in order to increase the anti-salmonella or anti-staphylococcal activity of CNFs, it is possible to combine chitosan with other antimicrobial agents such as ethylenediamine tetraacetic acid (EDTA, 0.2%) (Olaimat & Holley, 2015) and essential oils (Shahbazi & Shavisi, 2016), for a synergistic effect.

Table 5.1: Bacterial reduction rate (*R*) of CNFs against *E. coli*, *S.* Typhimurium, *L. innocua* and *S. aureus*, as quantitatively assessed by the CFU method, after 4 hr incubation at 37°C in PBS (pH 5.8).

	Reduction rate (%)			
Nanofiber webs	*E. coli*	*S.* Typhimurium	*S. aureus*	*L. innocua*
1 cm^2 CNFs	99.93 ± 0.5	96.81 ± 2.3	99.14 ± 1.8	99.90 ± 0,02
2.5 cm^2 CNFs	100 ± 0	98.97 ± 1,2	99.98 ± 0,5	100 ± 0

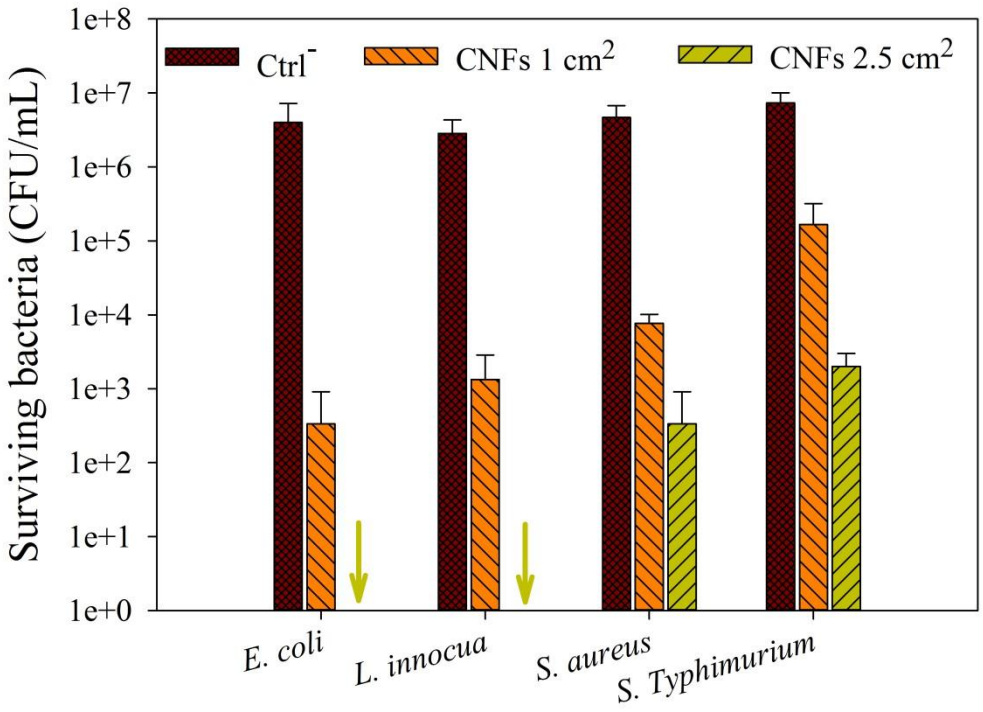

Figure 5.3: Antibacterial activity of CNFs against *E. coli*, *L. innocua*, *S. aureus* and *S.* Typhimurium, as evaluated by the dynamic CFU method after 4 hr incubation at 37°C in PBS (pH 5.8). The arrows point at the complete inhibition of bacterial growth (*R*=100%).

5.4.3 Proteins leakage

The release of intracellular proteins is an indication of membrane deterioration. Figure 5.4 shows SDS-PAGE patterns of released cytoplasmic soluble proteins from chitosan treated *E. coli* and *S. aureus*. In the case of *E. coli*, the protein content in the cell-free supernatant was similar to that of the positive control ($Ctrl^+$) that refers to bacterial suspension after cell lysis treatment. This result indicates that the effect of CNFs was instantaneous (in the first hour of treatment) and almost fully completed since all intracellular proteins were released to the extracellular medium, as judged by the comparison between the CNF-treated and the chemically lysed samples. For *S. aureus* bacterium, the effect was gradual. The electrophoresis pattern showed that the intensity of the bands increased with time, indicating that protein leakage was longer and progressive. When compared to the positive control ($Ctrl^+$), the effect of CNFs was incomplete and several bands did not appear even after 4 hr exposure. This indicates that lots of proteins remained in the cytoplasm of living cells. These results suggest that chitosan plays an active role in membrane

permeabilization. However, the observed antibacterial effect of CNFs on membrane damage, as reported by protein release was more pronounced in the case of *E. coli* than *S. aureus*, suggesting a higher susceptibility of *E. coli*, as reported in another study (Arkoun, Daigle, Heuzey, & Ajji, 2017).

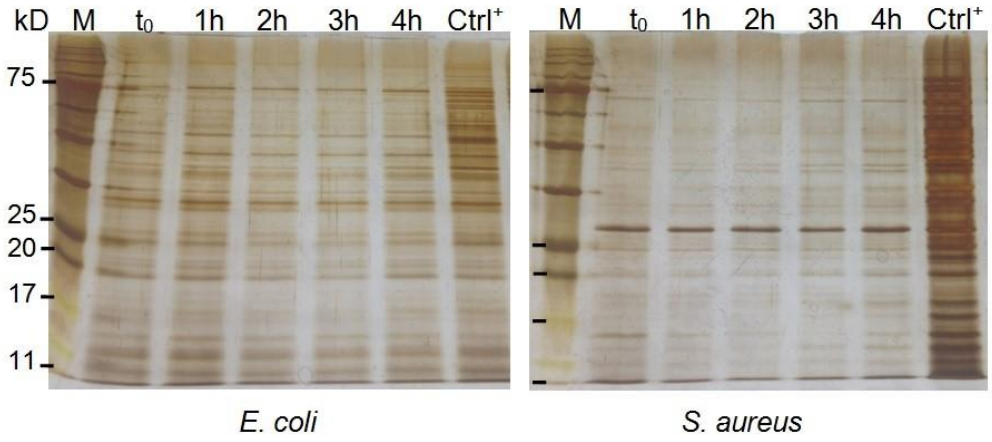

Figure 5.4: SDS-PAGE patterns of released intracellular proteins from CNF-treated *E. coli* and *S. aureus*, after 0, 1, 2, 3 and 4 hr contact time at 37°C in PBS. $Ctrl^+$ refers to total proteins chemically extracted after treatment of cells with a lysis solution containing 50 µl chloroform and 25 µl SDS (0.5% v/v).

5.4.4 DNA leakage

The release of bacterial genomic DNA in the supernatant was detected by PCR amplification of the *rrnB* gene (16S) for *E. coli* (Figure 5.5). The additional step of pH adjustment to neutrality mentioned in the methodology was necessary to hinder complexation of DNA with CNFs. Otherwise, no trace of the former could be detected. Detection of DNA in the extracellular medium (supernatant) was a consequence of the disruption of membrane permeability caused by CNFs (Figure 5.5A and 5B). In contrast, no DNA was detected in the extracellular medium of untreated sample ($Ctrl^-$, Figure 5.5D), which was synonymous with membrane integrity. The observed brightness at the loading spots of the treated samples was probably due to a deposition of small cationic chains of CS itself, which did not migrate towards the cathode. This can be also attributed to a deceleration of the electrophoretic mobility of genomic DNA caused by the chelation effect of chitosan, as suggested by Xing, Chen, Liu, Cha, and Park (2009b). Negatively charged phosphate groups present in nucleic acids such as DNA and RNA might be an intracellular target for CS and contribute to its interaction with bacterial cells. This conjecture was verified when CS was deprotonated (at neutral pH) in order to prevent CS-DNA

complexation. As a consequence, genomic DNA was detected both qualitatively and quantitatively. These results point out that the leakage of bacterial DNA would not occur without membrane perforation and strongly suggest a membranolytic effect in CNFs' mechanism of action. The concentrations of released DNA after exposure to CNFs, as measured using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific), after PCR were 18.2, 19.5, 20.9, 60.2 and 172.3 ng/µl, after 0, 1, 2, 3 and 4 h exposure times, respectively. Quantification of released DNA from CNF-treated *E. coli* clearly indicates that genomic DNA could be detected in the extracellular medium and its concentration was proportional to the contact time between *E. coli* and CNFs.

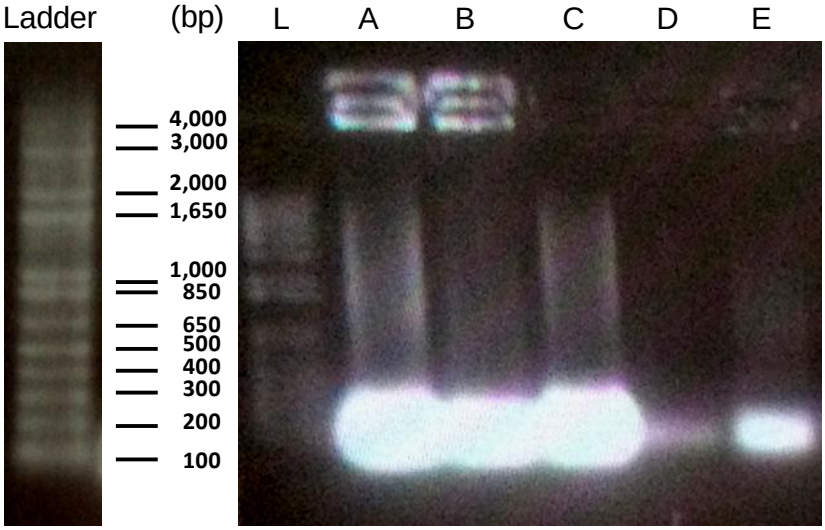

Figure 5.5: Agarose gel electrophoresis of released genomic DNA from CNF-treated *E. coli* after A: 4 hr and B: 24 hr contact time. Samples C and E refer to $CtrlL^{+}$ and $CtrlH^{+}$ positive controls of *E. coli* DNA after chemical and heat treatment of bacterial cells, respectively. Sample D refers to negative control of genomic DNA extracted from untreated bacterial cells. L refers to ladder's fragments whose molecular weights are given in base pair (bp).

5.4.5 Release of intracellular β-galactosidase enzyme

The release of cytoplasmic β-galactosidase (β-gal) was also an evidence of membrane permeabilization. Figure 5.6 shows the release of β-gal enzyme from *E. coli* after different contact times with CNFs. The results revealed that negative controls of untreated bacteria (black squares) showed no enzymatic activity, which was an indication of membrane integrity. When CNFs were added to the bacterial suspension, a progressive time-dependant enzymatic activity was observed (red circles), a consequence of membrane lesion. However, results demonstrated

that it was not possible to reach the maximum expected level of released β-gal from chemically lysed cells (~ 20 β-gal units). This suggests that the antibacterial effect of CNFs was not completed and the release of the enzyme is a longer process occurring after death and lysis of the cell. These results reasonably demonstrate the ability of CNFs to permeate bacterial membrane and coincide with the findings of Tao et al. (2011), who reported similar results for CS solutions.

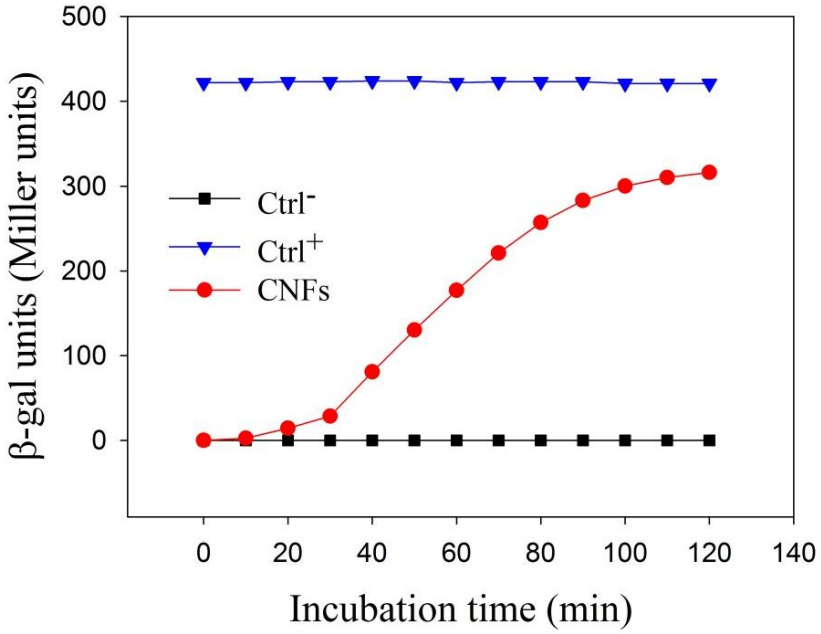

Figure 5.6: Release of cytoplasmic β-galactosidase (β-gal) enzyme from *E. coli* DH5 hxt 55632–*Lac* Z^+, after different exposure time to CNFs. $Ctrl^-$ (negative control) refers to the level of released β-gal in the absence of CNFs. $Ctrl^+$ (positive control) refers to the level of β-gal released by chemically lysed cells (prepared by adding 50 µl of chloroform and 25 µl of SDS to the culture)

5.4.6 Transmission electron microscopy (TEM) analysis of membrane permeabilization effect of CNFs

The effect of CNFs on membrane morphology and integrity was investigated by TEM (Figure 5.7). Untreated cells of *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) were intact and did not show any membrane lesion or anomaly (Figure 5.7a and 5.7e). After exposure to CNFs, a remarkable alteration of membrane integrity was observed. TEM images of exposed cells to CNFs revealed that after 10 min contact, both *E. coli* and *S. aureus* strains showed membrane permeabilization by perforation (Figure 5.7b and 5.7f). After 20 min exposure, both bacteria were leaking cytosolic components (Figure 5.7c and 5.7g). However, membrane detachment occurred

only in *E. coli* (Figure 5.7d). Gradual membrane detachment from the cell wall of *E. coli*, and shrinkage of the cytoplasm was observed after 30 min contact time with CNFs, as pointed by the blue arrows (Figure 5.7d). This detachment of the plasma membrane was due to desorption of the cytosol, subsequent to leakage of intracellular compounds (Figure 5.7c), making cells look transparent and empty (Figure 5.7d). After 30 min contact time, the cytoplasmic membrane of *E. coli* collapsed (Figure 5.7d) and *S. aureus* cells were completely disintegrated (Figure 5.7h). Adsorption of molecules to bacterial cell walls, of both *E. coli* and *S. aureus* was also observed and was proportional to contact time (Figure 5.7b, 5.7c and 5.7d). This might be due to (1) the release of intracellular components that can attach to the surface of bacteria, reflecting local cell rupture, or (2) to small soluble CS chains surrounding the bacterial cells *via* electrostatic and hydrophobic interactions, or (3) both possibilities.

A simple visual inspection of the CS/PEO nanofiber mats, before and after the antibacterial tests indicated that the fibers were stable after 48 hr at 37°C, pH 5.8 in PBS. This suggests that resolubilization was only partial as the mats remained intact. However, as nanofibers contain PEO, which is soluble in water, a certain solubility of PEO is expected. In addition, due to the pH of the medium (5.8), chitosan may solubilize partially, as verified by Ardila, daigle, Heuzey, and Ajji (2017). Consequently, both the released chitosan in the medium and the one remaining in the nanofiber mats may contribute to the antibacterial effect of the CNFs. The second conjuncture coincides with the findings of other authors (Chung et al., 2004; Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001), who studied the adsorptive characteristics of bacterial cells to chitosan solutions. This suggests that the mechanism of action of CNFs may be also due to partial resolubilization of CS in the media, even though visually the mats looked intact after 48 hr in PBS or LB. Short CS chains might, thereby, penetrate the cell wall and perforate the plasma membrane, while longer chains could enclose bacteria and prevent cell exchange with the extracellular medium. Accordingly, Figure 7b, c, d, and f, show that CS formed an impermeable envelope surrounding the bacteria which might block the absorption of essential elements into the cells (Choi et al., 2001; Eaton, Fernandes, Pereira, Pintado, & Malcata, 2008).

Ultimately, it can be inferred that the bactericidal effect of CNFs may be the result of membrane perforation. Our results are in agreement with those of other authors (Tao et al., 2011; Xing et al., 2009a), who observed membrane perforation of *E. coli* caused by CS solutions and particles, respectively. However, our experiments conducted on CNF-treated *E. coli* (Gram-negative) and

S. aureus (Gram-positive), revealed various surface characteristics and cell stages in response to treatment with CNFs.

This suggests that the mechanism of action of CNFs is a complex combination of different bactericidal effects that can occur at different stages: (1) CNFs inhibit bacterial growth through membrane pervasion and perforation, (2) partly resolubilized CS chains can kill bacteria by causing membrane rupture and/or suppressing cell exchange and nutrient uptake, (3) CS nanofibers and/or solutions can cause osmotic stress by chelating trace elements such as metallic ions, essential to bacterial growth. However, the common mechanism behind these different modes of action is undeniably due to the protonated functional groups of CS. The results clearly demonstrate that CNFs' bactericidal effect involves permeabilization of bacterial membrane with pore formation, contrary to what has been reported so far. However, no evidence of penetration of the membrane can be inferred, even though pore formation assuredly occurred. The next challenge should aim at clarifying the molecular mechanisms behind the bactericidal activity of CNFs and identifying the membrane elements and metabolic pathways involved in the internalization of chitosan into the bacterial cell wall. These further studies will not only be critical for the application of such materials in food packaging, but also for the prevention of outbreak of resistance phenomena toward chitosan.

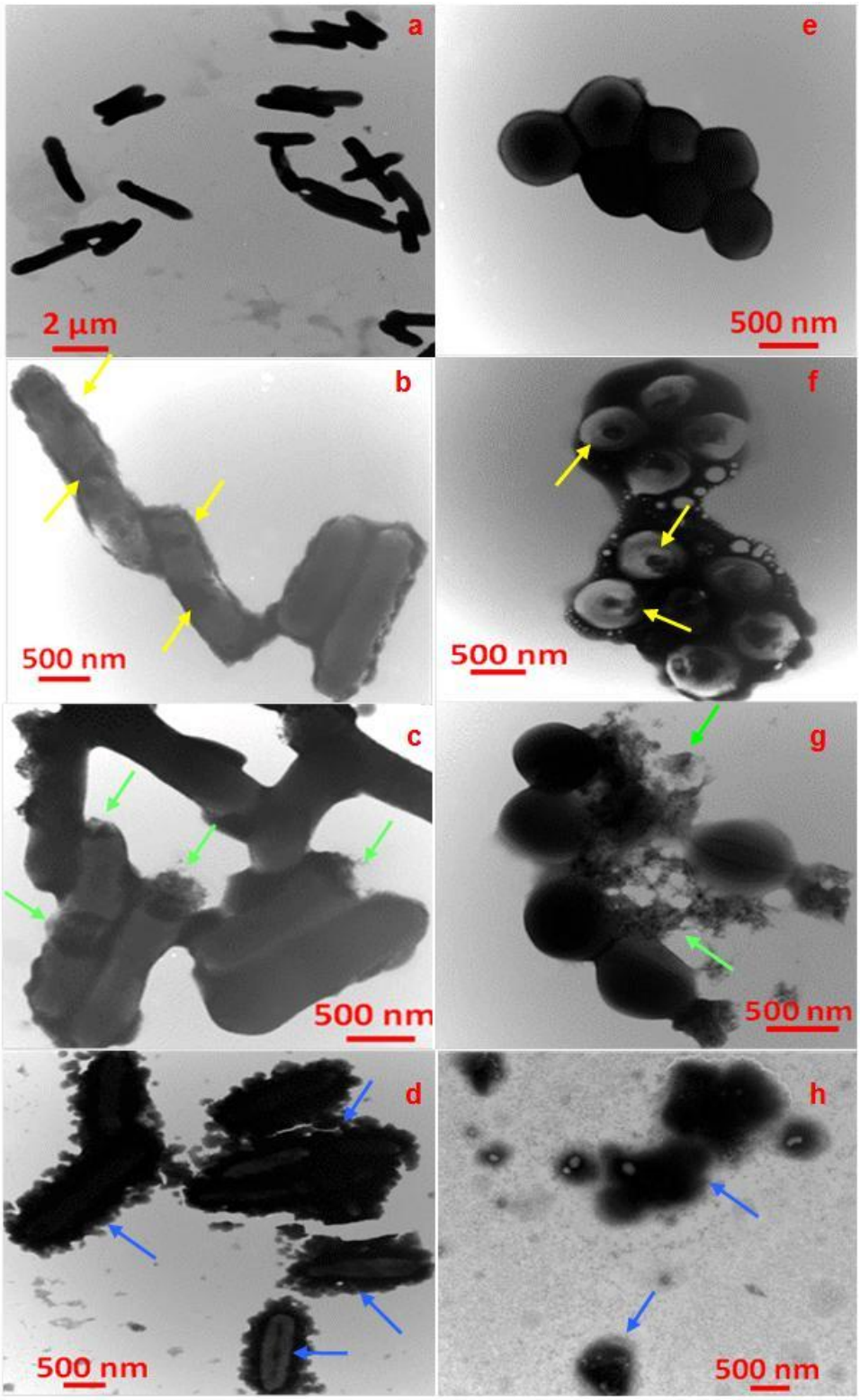

Figure 5.7: Transmission electron microscopy (TEM) micrographs of a, b, c, d: t_0, 10, 20 and 30 min exposure of *E. coli* cells to CNFs, and e, f, g and h: t_0, 10, 20 and 30 min exposure of *S. aureus* cells to CNFs respectively. The yellow, green and blue arrows respectively point at membrane perforation, leakage of cytosol and cell lysis.

5.5 Conclusions

The results of this study show that the antibacterial activity of chitosan nanofibers (CNFs) can be attributed to membrane disruption and perforation. Consequently, this resulted in the leakage of intracellular components such as proteins and nucleotides. The bioavailability of NH_3^+ functional groups on CNFs favored and maximized cell adhesion and attachment to the surface of the mats. The model established here, regarding CNFs' mode of action suggests that bacteria migrate to the surface of the nanofibers and not the reverse. Since bacteria use adhesion and attachment surfaces to better grow and multiply, CNFs showed the ability to efficiently attract and trap bacteria through electrostatic interactions, on account of their large surface-to-mass ratio and high porosity. Our results also suggest that adsorption of CS to the bacterial surface is the first step in CNFs' mechanism of action, followed by membrane perforation, leakage of cytosolic compounds and finally cell lysis and disintegration. Nevertheless, it is not excluded that part of the antibacterial activity might be due to partial dissolution of the nanofibers, making chitosan available in solution. As promising practical application, CNFs can be used as part of active food packaging in order to extend the shelf life of food products along with preventing spoilage by bacteria such as *E. coli*, and foodborne diseases caused by *Listeria*, *Staphylococcus* and *Salmonella*.

Acknowledgements

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CHAPITRE 6 ARTICLE 3: CHITOSAN-BASED NANOFIBERS AS BIOACTIVE MEAT PACKAGING MATERIALS

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6.1 Abstract

Shelf life and safety of minimally processed food is crucial for both consumers and the food industry. This study investigates the *in vitro* and *in situ* efficiency of electrospun chitosan-based nanofibers (CNFs) as inner part of a multilayer packaging in maintaining the quality of unprocessed red meat. Activated CNF-based packaging (CNFP) were obtained by direct electrospinning of chitosan/poly(ethylene oxide) solutions on top of a conventional multilayer food packaging. The electrospinning solutions were firstly characterized at the molecular level, mainly in terms of zeta potential and viscoelastic properties and the evolution of the conformational structure was correlated to the nanofiber formation process. The oxygen and water vapor barrier properties of CNF-based (CNFP) meat packaging were also investigated. The *in vitro* antibacterial activity of CNFs was determined against *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Listeria innocua*, bacteria commonly incriminated in the alteration of food products. The efficiency of the CNFP materials against meat spoilage by *E. coli* was also assessed. Our results indicate that the electrospinning of CS is a multifactorial process and fiber formation requires the choice of a good solvent, high electrical conductivity, moderate surface tension, optimum viscoelastic properties and sufficient chain

flexibility and entanglement. The results also indicate that all the tested bacterial strains were significantly sensitive to the action of CNFs. The *in situ* bioactivity against *E. coli* showed the potential of CNFP as bioactive nanomaterial barriers to meat contamination by extending the shelf life of fresh meat up to one week.

Keywords: chitosan nanofibers, electrospinning, antibacterial food packaging, shelf life, meat.

6.2 Introduction

Each year, more than one billion tons of food is wasted around the globe while still being perfectly edible. This represents one third of all food produced for human consumption and losses which, in 2014 reached $750 billion. [1] According to Stuart, [2] the amount of food wasted is enough to feed four times the number of persons who suffer from hunger in the world. In Canada, food losses reached $31 billion in 2014 and, according to data from the "Food Waste in Canada" study conducted by the Value Chain Management Center organization, [3] 47% of wasted food originated at home. In the province of Quebec, it is estimated that a household wastes nearly $800 of food per year, twice as much as in the US. [2] Besides, foodborne diseases resulting from consumption of contaminated food represent a serious public health issue in North America. In the US, millions of foodborne diseases occur and are reported every year. The costs associated with outbreaks of foodborne illnesses represent $152 billion and in Canada would be about a tenth of that. [4] According to the Ministry of Agriculture, Fisheries and Food of Quebec (MAPAQ), in 2012-2013 nearly 90% of confirmed or probable food poisoning was of microbiological origin. [5] Obviously, faced with the challenges of food spoilage and food poisoning that directly affect public health, economic and environmental issues, authorities must take adequate measures to minimize their influence.

One customary way to overcome these two problems is to extend the shelf life of food, for example through active packaging. The use of bioactive food packaging to extend shelf life and guarantee safety of perishable food, particularly those susceptible to microbial alteration, have gained considerable interest. [6-10] During the last few decades, substantial effort has been made by researchers studying packaging, materials and food processing as well as biotechnology to design active food packaging materials and satisfy consumer demand for both safe and preservative-free food products. Unlike passive packaging where the role is only to restrict exchanges of O_2, CO_2,

water vapor and aromatic compounds between the food and its local or external environment, bioactive packaging also provides antibacterial action [11]. Its ultimate function is to extend shelf life while ensuring safety of the food product.

The incorporation of antimicrobial agents into existing food packaging can protect food from microbial alteration and extend shelf life, reducing economic losses and health hazards due to foodborne pathogens. [12] Among the active compounds that have received attention recently are spices, herbs and their essential oils (EOs), [13-15] chitosan, [16] bacteriocins, [17] ethylenediamine tetraacetic acid (EDTA), [4] smoke antimicrobials, [18] metal oxides in the form of nanoparticles (e.g., zinc oxide (ZnO), titanium dioxide (TiO_2), silver (Ag), gold (Au)), [19] among others. The incorporation of spices, herbs and their EOs either to the packaging itself or directly into processed food has been reported. [10] However, alteration of organoleptic properties is sometimes unavoidable, often with a detrimental effect on the flavour and taste of the food product. Although the use of bioactive food packaging has been the subject of many scientific studies and reviews, [20-28] the reality of the market is different. Currently, there is no commercially available antibacterial food packaging. The potential beneficial effects of some bioactive compounds are generally well documented, however, their ecotoxicological impact has so far been seldom studied. [29,30] For example, some investigations have shown that there are many reasons to suspect that nanoparticles may have toxicological effects on biological systems. [31-33] Moreover, perception, trust and acceptance by the public of using such additives have a crucial impact on the achievement of technological progress. [34] In addition, uncertainty about possible migration or interaction between these additives and the food raises food safety concerns and may represent an obstacle to the marketing of such packaging technology. [21]

Chitosan, a modified natural carbohydrate polymer derived from fisheries industry by-products, has the advantage of being non-toxic, is compatible with biological systems and exhibits intrinsic antimicrobial properties. Using chitosan as a bioactive material has been the subject of several studies since 1980. [16,35-41] However, a limited number of studies have investigated the antimicrobial potential of chitosan in real food systems, with the majority focusing on chitosan solutions, films and coatings. [16] The use of chitosan in the form of solvent-cast films remains limited by its poor mechanical and barrier properties and low processing yield. On the other hand, solutions and coatings involve immersing the food in a chitosan-based solution containing a solvent, which may have the opposite effect, i.e. causing the early alteration of the food instead of

maintaining its sanitary safety. Nanofiber technology allows the fabrication of ultrathin non-woven mats with a substantially high surface area to volume ratio. Combined with the high porosity of the mats, nanofibers with antimicrobial, haemostatic, regenerative properties and water holding capacity can be obtained, depending on the base material. The resulting nanofiber mats can find promising applications in active packaging, biomedical areas and water filtration, among others. However, the electrospinning of CS is a multifactorial challenging process that requires a deep understanding of the electrospinnability of CS solutions at the molecular level. This issue may represent a limit regarding the use and exploitation of the advantages that nanofiber technology can offer. Several authors have investigated the effect of solution parameters (polymer concentration, surface tension, electrical conductivity and viscosity) on the fiber formation process. [42-47] However, the relationships between CS molecular characteristics, the evolution of its conformational structure during the fiber formation process still need to be addressed. In this work, the physicochemical and rheological characterization of CS-based solutions in terms of pH conditions, zeta potential and viscoelastic properties were correlated to the electrospinnability of CS for a better understanding of the fiber formation process. Then, a practical application of electrospun CNFs was designed. This study is the first to investigate the antibacterial efficiency of CNF-based materials when electrospun on top of an existing packaging material in maintaining the microbiological quality and safety of fresh red meat and improving its preservation under real conditions. To the best of our knowledge, this study is the first to investigate the antimicrobial potential of CNFs during storage with real food and examine their ability to reduce spoilage and loss of food, when part of an active packaging film.

6.3 Materials and methods

6.3.1 Materials

Chemicals. Chitosan (CS) with 95% degree of deacetylation (DDA), 50 kDa number average molecular weight (M_n) and a narrow polydispersity (grade obtained through enzymatic treatment), as specified by the supplier, was obtained from Ovensa Inc. (Toronto, ON Canada). Poly(ethylene oxide) (PEO), a co-spinning agent for chitosan, with a molecular weight of 600 kDa, and acetic acid (AcOH, glacial, 99.7%) were purchased from Fisher Scientific (Saint-Laurent QC, Canada). All materials were of analytical grade and used as received.

Culture media. Luria-Bertani (LB) broth was used as a growth medium for the selected bacteria. Cultures were diluted using phosphate buffer saline (PBS, pH 5.8, adjusted with 1 M HCl). LB agar (1.5%) was used as a solid medium for counting the surviving bacteria.

6.3.2 Microorganisms

Gram-negative bacteria *Escherichia coli* (DH5α) and *Salmonella enterica* serovar Typhimurium (SL1344), and Gram-positive bacteria *Staphylococcus aureus* (54-73) and *Listeria innocua* (ISPQ3284) were supplied by the Laboratory of Microbiology of Université de Montréal (Montreal, QC, Canada). Cultures were maintained at 4 °C prior to use, then transferred to a liquid culture medium and incubated at 37 °C for 24 h in an orbital shaker (New Brunswick Scientific, Edison NJ, US) to achieve an initial concentration of 10^9 colonies forming unit per milliliter (CFU/mL). Figure 6.7 in the Supporting Information illustrates morphological differences (Gram-type, means of motility, presence of flagella) among the tested bacteria. More details regarding the mechanism of action and the effect of CNFs on membrane integrity have been reported in another study [48].

6.3.3 Preparation of CS/PEO solutions

Chitosan (CS) and polyethylene oxide (PEO) electrospinning stock solutions (7 and 3 % w/v, respectively) were individually prepared by overnight dissolution of the respective polymer powder in aqueous acetic acid (AcOH) solutions at concentrations ranging from 10 to 90 % (v/v). CS/PEO blends were obtained by mixing the two polymer solutions under magnetic agitation for 4 h at different wt ratios ranging from 100 % PEO to 100 % CS. The solutions obtained were then immediately used in the electrospinning process

6.3.4 Preparation of CNFs and CNF-based packaging (CNFP) through electrospinning

CS/PEO nanofibers (CNFs) were prepared using the electrospinning process. Electrospinning of the blend solutions was performed using a horizontal homemade setup containing (1) a high voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, US), (2) a programmable pump (Harvard Apparatus, PHD 2000, Holliston, MA, US) to deliver the polymer solution at the required flow rate, and (3) a metallic rotating drum wrapped either with non-stick

aluminum foil or with the selected multilayer film to collect the nanofibers. The CNF-based packaging CNFP were prepared by direct electrospinning the CNFs on top of the multilayer packaging. A schematic representation of the homemade set up is shown in Figure 6.1. Optimal electrospinning process parameters were: flow rate of 0.5 mL/h, voltage of 20 kV and needle tip-to-collector distance of 20 cm. All experiments were conducted at room temperature (22 ± 1 ºC), relative humidity of 20-30 % and under atmospheric pressure. The collected electrospun nanofibers were dried overnight under a hood to allow complete evaporation of the solvent.

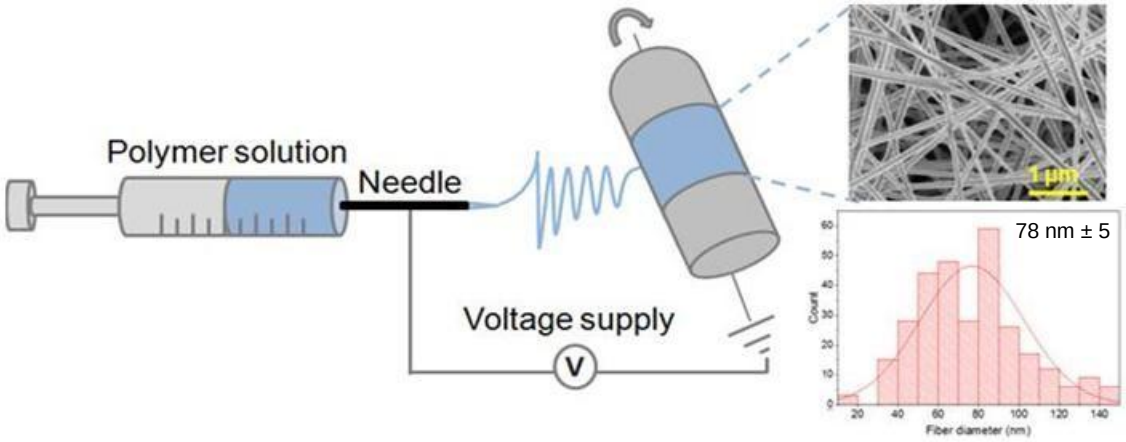

Figure 6.1: Schematic representation of the homemade electrospinning setup.

6.3.5 Characterization of the electrospinning solutions

6.3.5.1 Zeta potential

Zeta potential values of CS and CS/PEO solutions were determined by laser doppler velocimetry and phase analysis light scattering (M3-PALS) using a Malvern Zetasizer Nano ZSP instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The zeta potential was determined from the direction and velocity of the molecules in the applied electric field. The Smoluchowski model was used by the software to convert the electrophoretic mobility measurements into zeta potential values. All the samples were diluted (final concentration 0.01 % w/v) in deionized water and then put into a disposable folded capillary cell (DTS1060) for zeta potential measurements. The temperature of the cell was maintained constant at 25 ± 1 °C. Finally, the presented data were expressed as the average values of three independent sample measurements.

6.3.5.2 Rheology

The rheological properties of neat CS and PEO solutions as well as chitosan/PEO blends were studied in small amplitude oscillatory shear (SAOS) mode at 25 °C using a rotational rheometer (MCR 502, Anton Paar, Germany), equipped with a double Couette flow geometry. The viscoelastic behaviour of the solutions was firstly investigated. An amplitude of 5 % strain was found to be in the LVE region for both CS and PEO solutions as well as their blends. The elastic and loss moduli as well as damping factor (tan delta) were measured in frequency sweep tests ranging from 0.5 to 500 rad/s. All tests were conducted in triplicate and the results expressed as mean values of three independent samples.

6.3.6 Characterization of CNFs-based packaging

6.3.6.1 Scanning electron microscopy

The morphology of the electrospun chitosan nanofibers was examined with a field emission scanning electron microscope (FESEM, JEOL JSM-7600TFE field emission gamma, Hitachi Instruments Ltd., Tokyo, Japan), operated at 2 kV, as described by Moayeri and Ajji. [49] Samples were cut from the mat after 20 min electrospinning, mounted on the SEM stub without coating with gold and observed as collected on an aluminum foil. Fiber diameter distribution was evaluated using Image-Pro Plus software. Two hundred fibers, randomly chosen from three different samples were considered for the calculation of fiber diameter.

6.3.6.2 Barrier properties (permeability tests)

Water Vapor transmission rate (WVTR) of the neat and CNF-based packaging (CNFP) films, in accordance with the ASTM standard D-6701, [50] was measured using the MOCON Aquatran MG (Minneapolis, USA) permeability tester. WVTR tests were performed at 37°C, 100% relative humidity, 1 atm pressure and 100% nitrogen was used as the carrier gaz. The tests were ended when the water vapor flux changed by less than 1% during a 100 min test cycle. The reported data have been normalized (multiplied) by the films' thickness.

Oxygen transmission rate (OTR) of the neat and CNF-based packaging films was measured in accordance with the ASTM standard D-3985-81, [51] using the MOCON Oxtran 2/21 MD (Minneapolis, USA) permeability tester. OTR tests were performed at 25°C, 0% humidity and 1

atm pressure. A mixture of 98% nitrogen (N_2) and 2% hydrogen (H_2) was used as the carrier gas and 100% oxygen (O_2) was used as the test gas. The tests were ended when the oxygen flux changed by less than 1% during a 30 min test cycle. The reported data have been normalized (multiplied) by the films' thickness, including the thickness of the CNF mats.

6.3.6.3 *In vitro* antibacterial activity

The antibacterial activity of electrospun chitosan-based nanofibers (CNFs) was evaluated *in vitro* against *E. coli*, *S. aureus*, *L. innocua* and *S.* Typhimurium, bacteria frequently incriminated in food alteration and poisoning. TEM images of the selected bacteria are shown in Figure 6.7. Samples of 1 cm^2 and 2.5 cm^2 swatches of CNFs were prepared in aseptic conditions. Bacteria were put in contact with CNFs in 5 mL bacterial suspensions, prepared by diluting overnight cultures with PBS (pH 5.8) to reach 10^3 CFU/mL. It is important to mention that even though the CS grade used in this study was water-soluble, the resulting nanofibers were visually insoluble in aqueous media post-electrospinning due to solvent (acetic acid) evaporation during processing. Negative controls of bacteria suspended in PBS without CNFs were also prepared. All tubes were placed at 37 °C for 4 h incubation in an orbital shaker. Serial dilutions were performed and spread on agar plates incubated overnight at 37 °C – optimal temperature for bacterial growth – for further counting of survivors. All tests were conducted in triplicate.

6.3.6.4 *In situ* antibacterial activity of CNFs

In order to assess the antibacterial activity of CNFs under real conditions, meat preservation tests were performed. Two days after slaughter of the veal, fresh meat cubes (10 g) were cut under aseptic conditions. Samples were inoculated by immersion in a bacterial suspension of *E. coli* (10^3 CFU/mL) for 30 sec. Excess liquid was removed by draining for 30 sec, followed by 3 min drying under a biologic hood. Inoculated meat samples were then packed in a CNF-based packaging (CNFP) sealed under vacuum. The commercial multilayer food packaging used in this study was selected for its good mechanical and barrier properties. The film (sample labelled NP for neat packaging) was prepared using the co-extrusion process of poly (ethylene terephthalate) (PET) and ethylene vinyl alcohol (EVOH) and was provided by ProAmpac, Terrebonne, QC, Canada. A negative control ($Ctrl^-$) of inoculated meat, and inoculated meat wrapped in the neat conventional packaging (NP) as positive control ($Ctrl^+$), were also prepared under the same aseptic conditions. All samples were stored at 4 °C for further analysis. After 7 day storage,

samples were unwrapped and placed in 40 mL PBS. Surviving bacteria were collected by grinding the meat cubes with a laboratory tissue grinder in order to separate bacteria from the surface of meat tissue. After serial dilution, samples were spread on top of LB agar plates and incubated at 37 °C for 24 h for further counting of the surviving bacteria.

6.4 Results and discussion

6.4.1 Zeta (ζ) potential of CS-based solutions

Figure 6.2a and 6.2b respectively present the charge density of CS and CS/PEO solutions as reported by ζ potential measurements, as a function of (a) pH (AcOH strength) and (b) CS/PEO wt % ratio. The ζ potential of neat CS solutions showed a decreasing trend (from 76 until reaching 0 mV) due to the deprotonation of the amino groups on CS chains, as the pH increased from 2 to 7 or when the acid (AcOH) strength decreased from 50 to 0 % (v/v). Note that the maximum value of the ζ potential (76 ± 2 mV) was reached at pH 2 (equivalent AcOH content of 50 %). At pH values lower than 2 (pH 1, 90 % AcOH) a decrease of the ζ potential with respect to pH 2 was denoted. A saturation point was probably reached, as illustrated by the maximum (Figure 6.2a). This behavior may be explained in four postulates as follows; (1) At moderate low pH values [pH 2-6], the equivalent of acidic to weakly acidic conditions (50-0.25 % v/v AcOH), the amine functions of CS were progressively getting deprotonated and converted from $–NH_3^+$ to $–NH_2$; (2) At higher pH values close to neutrality, getting closer to its p*K*a (6.2-6.5), CS lost its polycationic character, again due to the deprotonation of its amino groups; (3) In neutral conditions (pH 7 > p*K*a), CS chains were completely deprotonated as judged by the zero charge density (ζ potential = 0 mV). This gradual deprotonation induced conformational changes and self-agglomeration of CS chains (pH 6) until complete precipitation at pH 7 (data not shown), as previously reported by others; [52,53] (4) Interestingly, at very high acid strength (pH 1, 90 % AcOH), CS chains being fully protonated, the excess acid could be responsible for the neutralization of cationic groups by charge screening. This phenomenon is an indication of the saturation of the cationic groups of CS, therefore suggesting a protonation threshold at which CS chains are fully protonated. Some authors have quantified this threshold by calculating a charge parameter λ, a protonation constant log *K* or the degree of protonation α. These parameters are

proportional to the charge density and strongly dependent on pH, DDA, type of acid and ionic strength. [54-58]

A variation of the CS/PEO content (ranging from neat chitosan to neat PEO) also affected the ζ potential and resulted in an increase from almost 20 to 57 mV (Figure 6.2b). In other words, the addition of PEO caused a gradual decrease of the charge density as reported by the lower ζ potential values. Flexible PEO chains can mask some positive charges on CS by interacting with its rigid chains through hydrogen bonding between hydroxyl and amino groups on CS and ether ones on PEO, [46] therefore leading to the observed decrease in ζ potential.

Since the functionality of CNFs is strictly governed by the protonation of the amine functions, these results provide a better understanding of the antibacterial activity of CNFs. Indeed, below chitosan p*K*a (up to a pH of 6), the global charge of CS is positive and CNFs exhibit strong bactericidal properties. Conversely, in neutral pH conditions, the corresponding ζ potential being 0, the antimicrobial activity of CNFs is completely lost. [59]

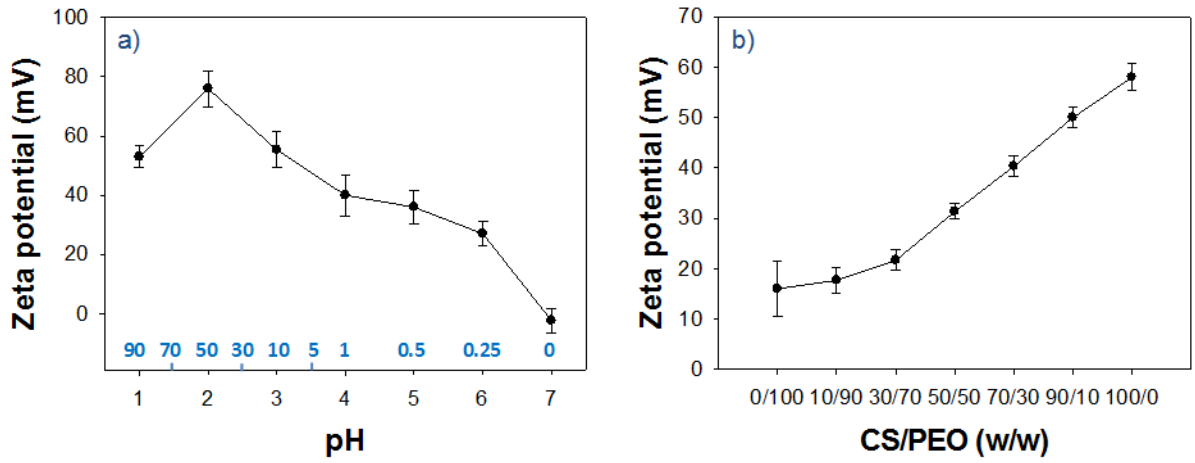

Figure 6.2: Zeta (ζ) potential of chitosan solutions (0.1 % w/v) as a function of a) pH and b) chitosan (CS)/PEO weight ratio at 50 % AcOH, pH 2. The blue scale refers to the AcOH content in v/v %.

6.4.2 Rheological behavior of CS-based solutions

This study is the first to investigate the role of CS chain viscoelasticity on the fiber formation process. Figure 6.3 presents the damping factor (tan delta) of CS, PEO and CS/PEO electrospinning solutions in relation with the AcOH content (v/v %) as well as CS/PEO ratio. The complex viscosity data of the same solutions can be found in the Supporting Information (Figure 6.8). The results of the damping factor (tan delta) measurements indicate that the elasticity of CS

solutions increased with the acid strength. It can be seen that the 90 % AcOH solution differs from the rest of the curves and showed the highest elasticity in comparison with the other solutions (Figure 6.3a). This behavior may be explained by the lower hydrodynamic volume expansion of CS molecules due to less electrostatic repulsion (charge screening), which favored a more flexible conformational structure of CS chains as supported by the ζ potential results above. Similarly, tan delta of PEO solutions was also affected by the solvent strength (Figure 6.3b). Indeed, the elasticity of the solutions markedly increased with the AcOH content, with the highest elasticity (as reported by the lowest tan delta) being attributed again to the 90 % (v/v) AcOH PEO solution (Figure 6.3b). Figure 6.3c reports the tan delta results of CS/PEO blends as a function of the CS/PEO wt ratio. Although the addition of PEO had little effect on tan delta of CS/PEO blends, the global trend was that the elasticity of these systems was improved by the addition of small amounts of PEO, *i.e*. 90/10 CS/PEO wt ratio in comparison with the neat CS solution. This result suggests possible interactions between CS and PEO, facilitated by the high affinity of the two polymers for each other.

The addition of PEO also led to a decrease in the complex viscosity of the blends (Figure 6.3) and the strong hydrogen bonds between the hydroxyl and amino groups on chitosan and the ether groups on PEO are believed to be the main reason for this decrease in viscosity. [46] Hence, PEO could break the inter- and intra- chain molecular interactions between CS chains via new interactions with PEO, thus lowering the stiffness of CS chains as proposed by others. [60] This finding is in line with the results of ζ potential of CS/PEO blends that strongly suggested that flexible PEO chains can interact with the rigid CS molecules. This theory is supported by the observed decrease in ζ potential with the addition of PEO. A similar effect could be obtained by the use of strong solvents such as trifluoroacetic acid (TFA) in order to lower the rigidity of CS solutions by breaking the CS inter- and intra- chain interactions (Figure 6.9). However, the toxicity of this type solvent excluded its use in applications such as food packaging. The non-toxic character of the material prepared here makes it particularly attractive, given that deliberate food–packaging interaction is the condition and the very definition of bioactive packaging.

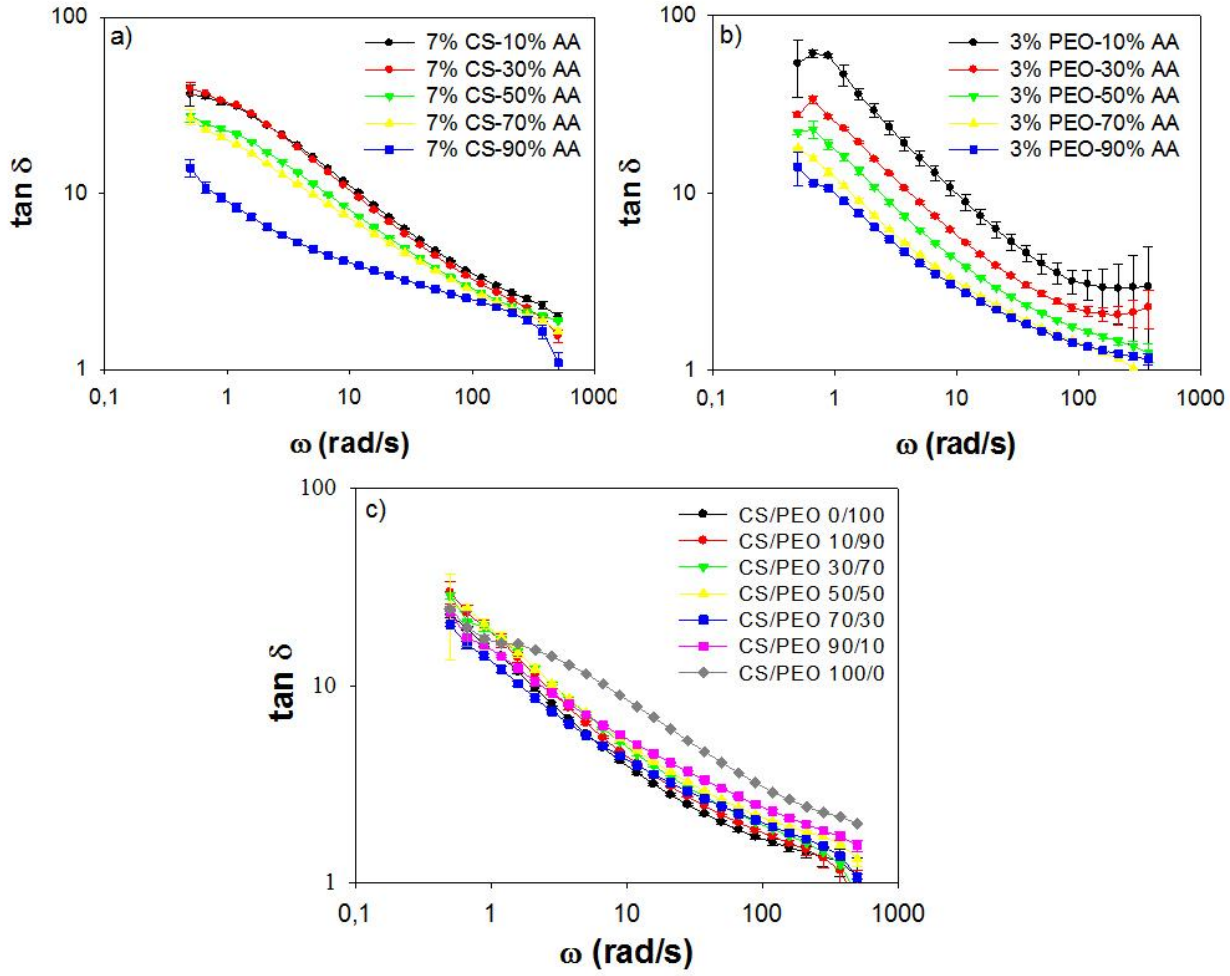

Figure 6.3: Viscoelastic properties (tan delta) versus angular frequency) of: (a) 7 wt % CS, (b) 3 wt % PEO and (c) CS/PEO blend solutions in small amplitude oscillatory shear (SAOS) at 5 % strain and 25 °C.

6.4.3 Scanning electron microscopy of CNFs

The morphology of the electrospun CS/PEO nanofibers (CNFs) is shown in Figure 6.4. Although the solvent used in this study was aqueous acetic acid (AcOH), a natural acid safely used in the food industry (main ingredient of vinegar), the utilisation of minimal concentrations of this solvent is preferable. Figure 6.4A reports the effect of the solvent strength (AcOH content) on the electrospinnability of CS and nanofiber formation. The results indicate that increasing the acid strength from 1 to 90 % v/v improved the nanofiber structure and enabled homogeneous and beadless fiber formation. High AcOH contents facilitated the electrospinnability of CS-based solutions by lowering the surface tension (Figure 6.10a). However, the side effect of that is that the electrical conductivity decreased (Figure 6.10a) which led to an increase in fiber diameter of

CNFs with lower yields of nanofibers, as shown in Figure 6.4A. This negative effect was caused by lower solvent evaporation rate at high acid concentration. Consequently, a concentration of 50 % v/v AcOH was chosen as a compromise between (i) a moderate electrical conductivity (2 mS/cm) that ensures the necessary electrostatic repulsions for fiber initiation, (ii) a relatively low surface tension (39 mN/m, Figure 6.10b) to decrease the free energy of the system and prevent the breakup of the polymer jet into beads, (iii) sufficient positive charge density that ensures the required repulsive forces for jet formation and stability (ζ potential = 58 mV), (iv) adequate flow properties (zero shear viscosity η = 1.5 Pa.s, Figure 6.8) and good elasticity that enables the elongation of the polymer droplet at the exit of the needle and prevents the fragmentation of the filament that causes electrospraying and bead formation, and (v) a reasonable solvent evaporation rate allowing fiber stretching and reaching high yields of nanofibers.

In the case of neat PEO solutions (Figure 6.11), increasing the AcOH content from 0 (water) to 1 and 50 % (v/v) led to a structure transition from uniform nanofibers to electrosprayed nanobeads and rough nanofiber morphology, respectively, despite lower surface tension values (59 mN/m vs 39 mN/m for 0% vs 50% AcOH, respectively). This effect was most likely due to the lower electrical conductivity of the PEO solutions. Figure 6.4B shows the effect of CS/PEO ratio on the final morphology of the corresponding nanofibers. The use of PEO as a co-spinning agent was necessary to improve the fiber formation process and facilitate the electrospinnability of CS. As surface tension was not affected by the addition of PEO to CS solutions (Figure 6.10), the low yields of fibers at high proportions of PEO (10/90, 30/70 and 50/50 CS/PEO ratios, Figure 6.4Ba, 4Bb and 4Bc, respectively) was attributed to the low electrical conductivity of the corresponding solutions. Conversely, the addition of low content of PEO improved fiber formation with high yield of CNFs (Figure 6.4Bd, 6.4Be and 6.4Bf). As demonstrated by the results of damping factor and ζ potential analysis, the addition of PEO facilitated the electrospinnability of CS solutions by enhancing chain flexibility and entanglement, two parameters that are critical for the electrospinning process. Moreover, the decrease of the addition of PEO helped improving the elasticity and the elongation of polymer chains under the electrical field. On the other hand, it was necessary to use the maximum permissible content of chitosan in order to maximize the dose-dependent bactericidal effect of CNFs. The 90/10 CS/PEO formulation exhibited smooth and homogeneous nanofiber structure along with strong antibacterial activity. Consequently, this formulation was selected for further characterization and analysis.

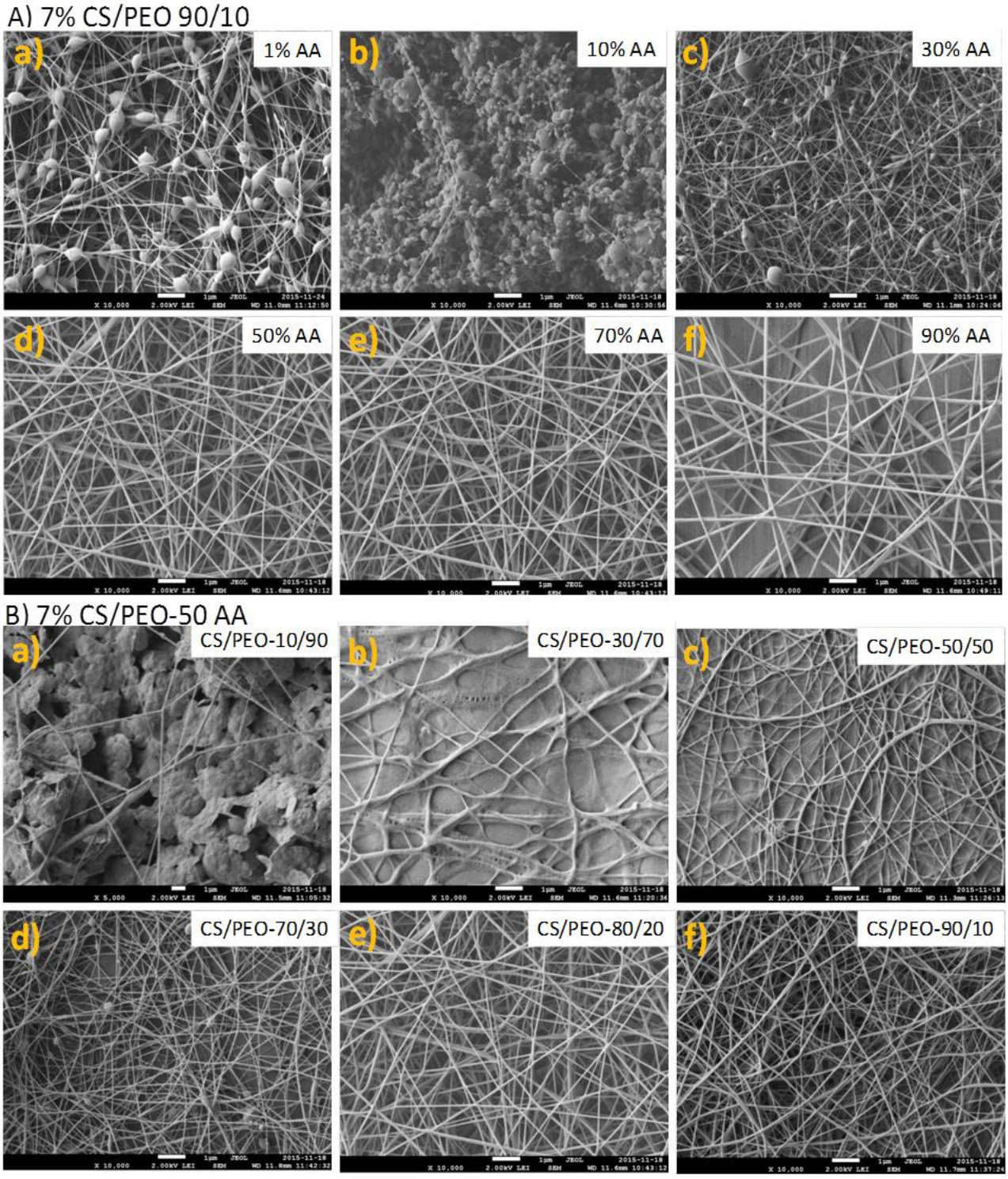

Figure 6.4: Nanofiber morphology of electrospun CS/PEO nanofibers (CNFs) made from A) CS/PEO (80/20) solutions in a) 1%, b) 10%, c) 30%, d) 50%, e) 70%, d) 90% AcOH and B) CS/PEO solutions in 50% AcOH with different CS/PEO wt ratio. A)10/90, b) 30/70, c) 50/50, d) 70/30, e) 80/20 and f) 90/10, respectively. AA refers to acetic acid (AcOH). All scale bars indicate 1 µm.

6.4.4 Barrier properties of CNF-based packaging

The barrier properties of the neat packaging and the CNF-based packaging were investigated in terms of oxygen transmission rate (OTR) and water vapor transmission rate (WVTR) and the results are presented in Table 6.1. As expected, the results indicate that CNFs (electrospun on top of the multilayer packaging film) did not have a significant effect on the OTR (1.29 vs 1.46 cc/(m^2.d) for neat and CNF-based packaging, respectively). By contrast, WVTR was raised by the presence of CNFs. This result was explained by the hydrophilic nature of CS and its remarkable water absorption ability, a characteristic that is responsible for its haemostatic properties. This advantage could be exploited to retain and absorb liquids released during the storage of certain foods including meat, chicken and fish, when CNFs are part of the multilayer packaging in direct contact with food. In this context, the water absorption capacity of CNFs may indirectly contribute to the antibacterial action of CNFs and thus inhibit or decrease bacterial growth by lowering the surrounding water activity, one of the most essential parameters for bacterial growth.

Table 6.1: Oxygen transmission rate (OTR) and water vapor transmission rate (WVTR) of neat and CNF-based packaging materials.

	Neat packaging	CNF-based packaging
OTR[1]	1.29 ± 0.3[a]	1.46 ± 0.3[a]
WVTR[2]	19.10 ± 2.3[b]	14.89 ± 1.2[c]

[1] cc/(m^2.d). [2] mg/(m^2.d).

[a, b, c] According to the Student t-test, means that do not share a letter in each group (OTR and WVTR) are significantly different ($p<0.05$).

6.4.5 *In vitro* antibacterial activity of CNFs and CNF-based packaging

Figure 6.5 shows the *in vitro* antibacterial activity of CNFs, quantitatively assessed by the colony forming unit (CFU) method against *E. coli*, *S.* Typhimurium, *L. innocua* and *S. aureus*. After 4 h contact at 37 °C in PBS (pH 5.8), CNFs showed significant reduction of bacterial growth (> 99.9 %). When CS content was increased (2.5 cm^2 instead of 1 cm^2 nanofiber mats), CNFs were able to completely stop the growth of *E. coli*, *L. innocua* and *S. aureus*, as shown by the arrows (Figure 6.5). In contrast, *S.* Typhimurium showed lower susceptibility to the action of CNFs. Nevertheless, a significant dose-dependent reduction of bacterial viability (one log) was still observed. It is evident that one way to increase the anti-*Salmonella* activity of CNFs would be to

combine the bactericidal action of chitosan with that of other antimicrobial agents such as ethylenediamine tetraacetic acid (EDTA, 0.2 %), for a synergistic effect [4]. These results suggest that CNFs are of potential value as a bioactive nanomaterial barrier to skin infection against *E. coli* and *S. aureus* and prevent food contamination by *E. coli*, *S.* Typhimurium, *S. aureus* and *L. innocua*. CNFs could help to maintain and even improve the safety of fresh perishable food such as meat during storage.

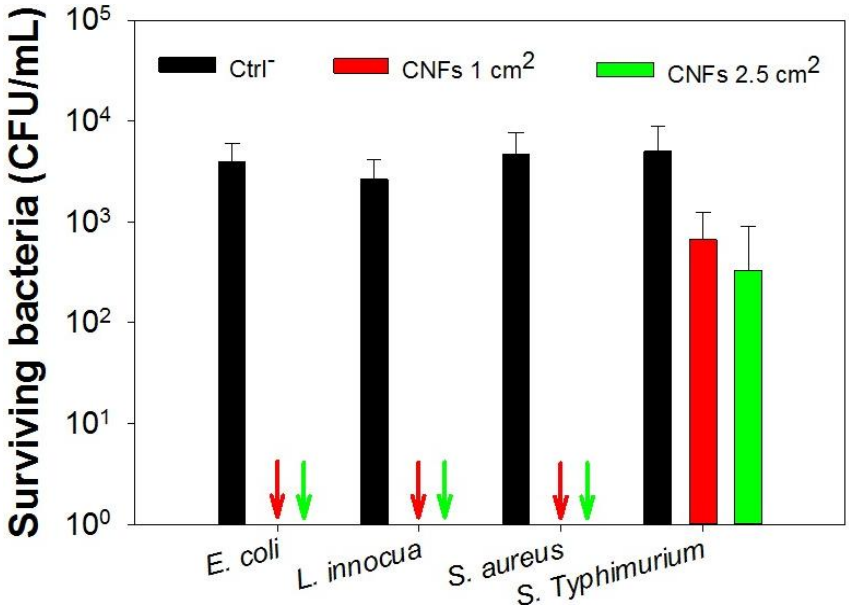

Figure 6.5: *In vitro* antibacterial activity of CNFs against *E. coli*, *L. innocua*, *S. aureus* and *S.* Typhimurium, as evaluated by the dynamic CFU method after 4 h incubation at 37 °C in PBS (pH 5.8) with an initial bacterial concentration of 10^3 CFU/mL. The arrows indicate total inhibition of *E. coli*, *L. innocua* and *S. aureus* growth.

6.4.6 Effect of CNFs-based packaging (CNFP) on meat preservation

The effect of electrospun CNF-based packaging (CNFP) was assessed under real conditions during refrigerated meat storage. Figure 6.6A reports the antibacterial activity of CNF-based packaging against *E. coli* (10^3 CFU/mL) at 4 °C, in comparison with the neat film. The results revealed that when contaminated meat was packed in a CNF-based packaging (CNFP, green bar), bacterial viability was reduced by 95 %, a bactericidal effect that allowed the extension of the shelf life of the tested meat to one week. It is not excluded that part of the antibacterial effect may be due to the decrease of the WVTR in the presence of CNFs, as shown by the permeability results. When the same contaminated meat was packed in the neat packaging (NP, red bar), a slight decrease in bacterial population was nevertheless observed in comparison with the negative

control ($Ctrl^-$, black rectangle). However, knowing that the initial bacterial concentration used to inoculate the meat was 10^3 CFU/mL, it is evident that the number of bacteria increased in both negative ($Ctrl^-$) and positive (NP) controls. The lower increase in bacterial concentration of inoculated meat wrapped in a conventional packaging (NP, red bar) is attributed to the good barrier properties provided by the commercial meat packaging. This packaging prevented the diffusion of oxygen and water vapor, two factors that are necessary for bacterial growth. Consequently, further alteration of the food was limited, however, this type of passive packaging was not able to eliminate the bacteria that were initially present in the sample. In contrast, CNFP, as active food packaging, eradicated 95% of the *E. coli* bacterial population, thus preserving the microbiological quality and safety of the meat. Figure 6.6B shows the appearance of unpacked meat and meat packed with CNFs before and after grinding. The results showed that organoleptic quality (appearance and smell) of the meat was altered in the absence of CNFs, while these properties were preserved when the meat was packed in the CNFP. These results indicate that CNFs are of potential value in active food packaging applications. CNFP help to maintain and even improve the safety of fresh red meat during storage, thus reducing food spoilage and foodborne illnesses along with extending shelf life of meat up to one week more.

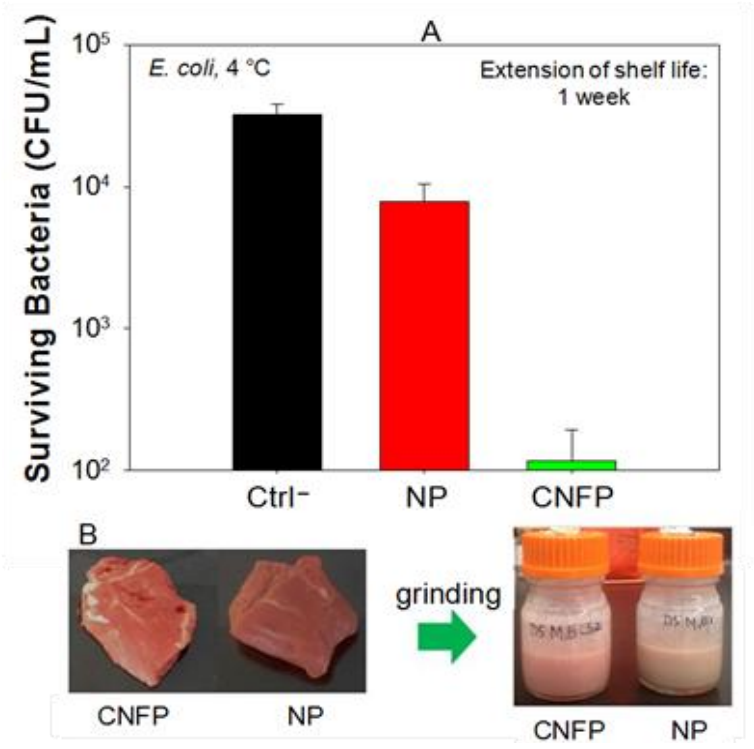

Figure 6.6: A: *In situ* antibacterial activity of CNFP against *E. coli* after 7 days storage at 4 °C. B: appearance of packed red meat with and without CNFP, before and after grinding.

6.5 Conclusions

In this study, highly antibacterial CNFs were successfully obtained. The physicochemical and rheological characterization of the electrospinning CS-based solutions deeply reflected the molecular characteristics of CS solutions such as charge density, chain flexibility and conformational structure for a better understanding of the fiber formation process. For the first time, a link was established between the effect of acid strength and the addition of PEO on the elasticity, charge density and flexibility of CS chains. This study is the first that investigates the efficiency of electrospun CNFs when coupled to an existing packaging in improving the quality and extending the shelf-life of a food product under real conditions. CNFP show an advantageous potential as antimicrobial packaging materials that preserve the quality and freshness of unprocessed or minimally processed and perishable foods such as meat, along with the extension of meat shelf life to one week.

CNFP thus represent promising biomaterials to reduce foodborne illness and spoilage, without the specific need for mixing antimicrobial additives with the food itself and without altering the organoleptic quality of the product.

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6.7 Supporting Information

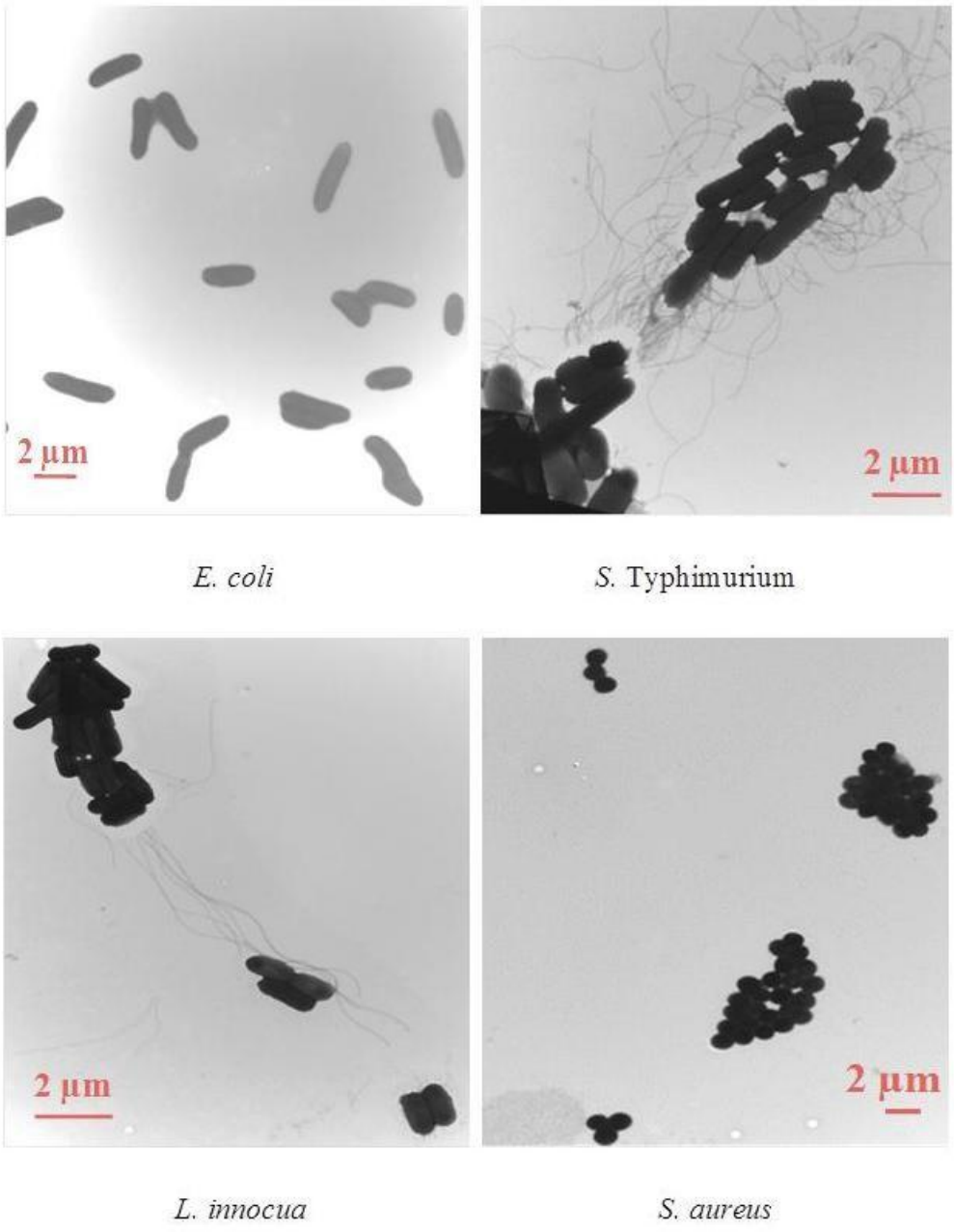

Figure 6.7: TEM images of the bacteria models used in this study.

The complex viscosity of CS solutions increased with the solvent (AcOH) strength (Figure 6.8a). This gain in viscosity can be explained by the expansion of the hydrodynamic volume of CS molecules due to high electrostatic repulsions between the protonated $–NH_3^+$ functions. However, at higher AcOH content (above 50 %), the complex viscosity of the solutions remained constant. This effect was attributed to the saturation of the cationic sites as CS chains were fully protonated, confirming the ζ potential results. At strong AcOH content (90 %, pH 1) the complex viscosity remained constant at low angular frequencies. However, at high frequencies, the shear thinning behavior was more pronounced. This effect was interpreted as a consequence of the disentanglement of CS chains due to a more flexible conformational structure, facilitated by the screening effect of the excess of acid. This result is supported by the decrease of the ζ potential at high acid content.

The same trend with a sharp increase in the complex viscosity was observed for neat PEO solutions. This behavior was attributed to the strong interactions between the ether groups of PEO and the hydroxyl ones of acetic acid, which allowed the expansion of PEO chains in acidic conditions, resulting in a remarkable increase of the complex viscosity (Figure 6.8b). Figure 6.8c reports the complex viscosity of CS/PEO blends as a function of the CS/PEO wt ratio. Indeed, the addition of PEO led to a decrease in the complex viscosity of the blends. As demonstrated by the ζ potential and conductivity measurements, PEO can decrease the hydrodynamic volume expansion by masking the positive charges of CS chains and lessening the electrostatic repulsions.

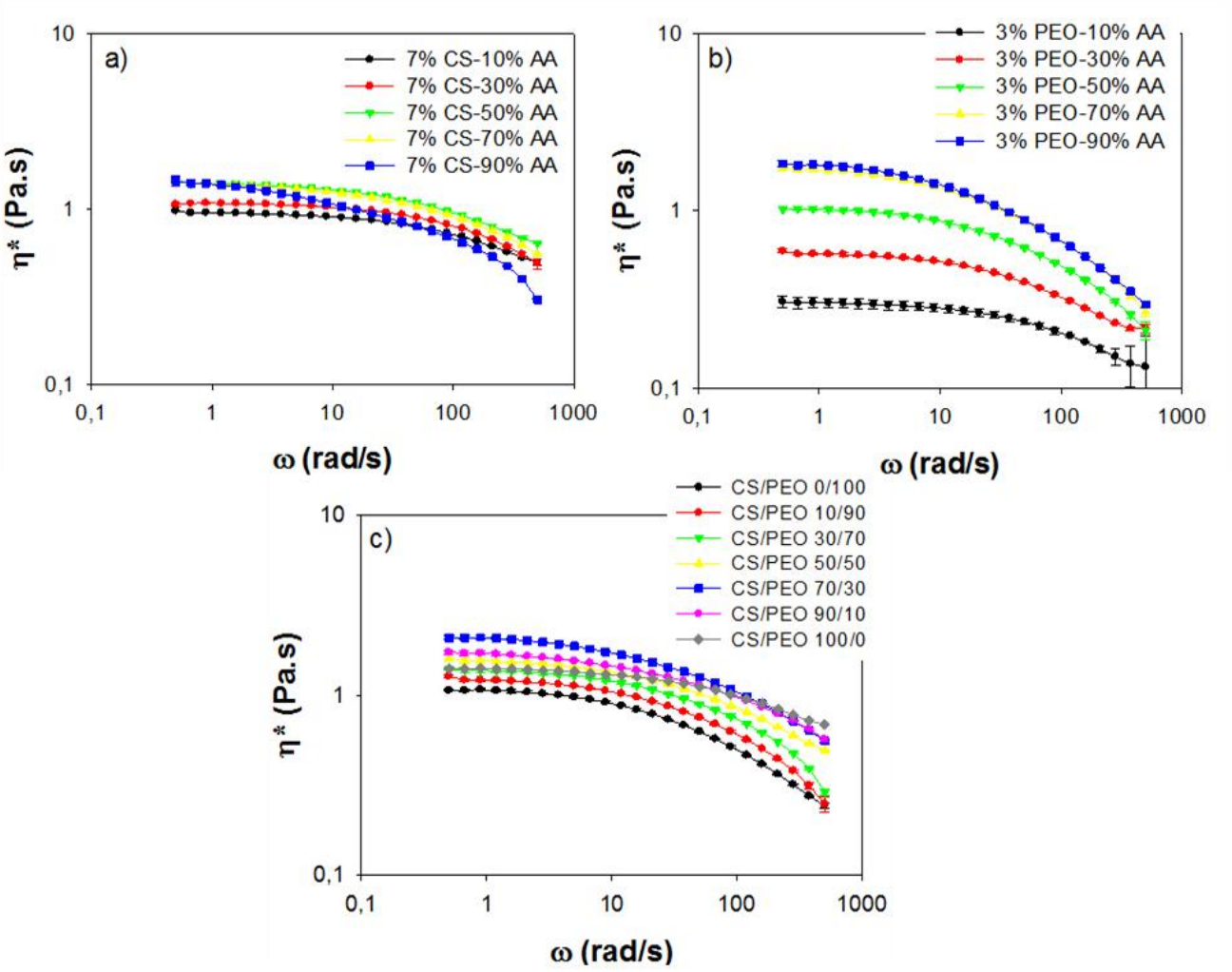

Figure 6.8: Complex viscosity versus angular frequency) of: (a) CS, (b) PEO and (c) CS/PEO blend solutions in small amplitude oscillatory shear (SAOS) at 5 % strain and 25 °C.

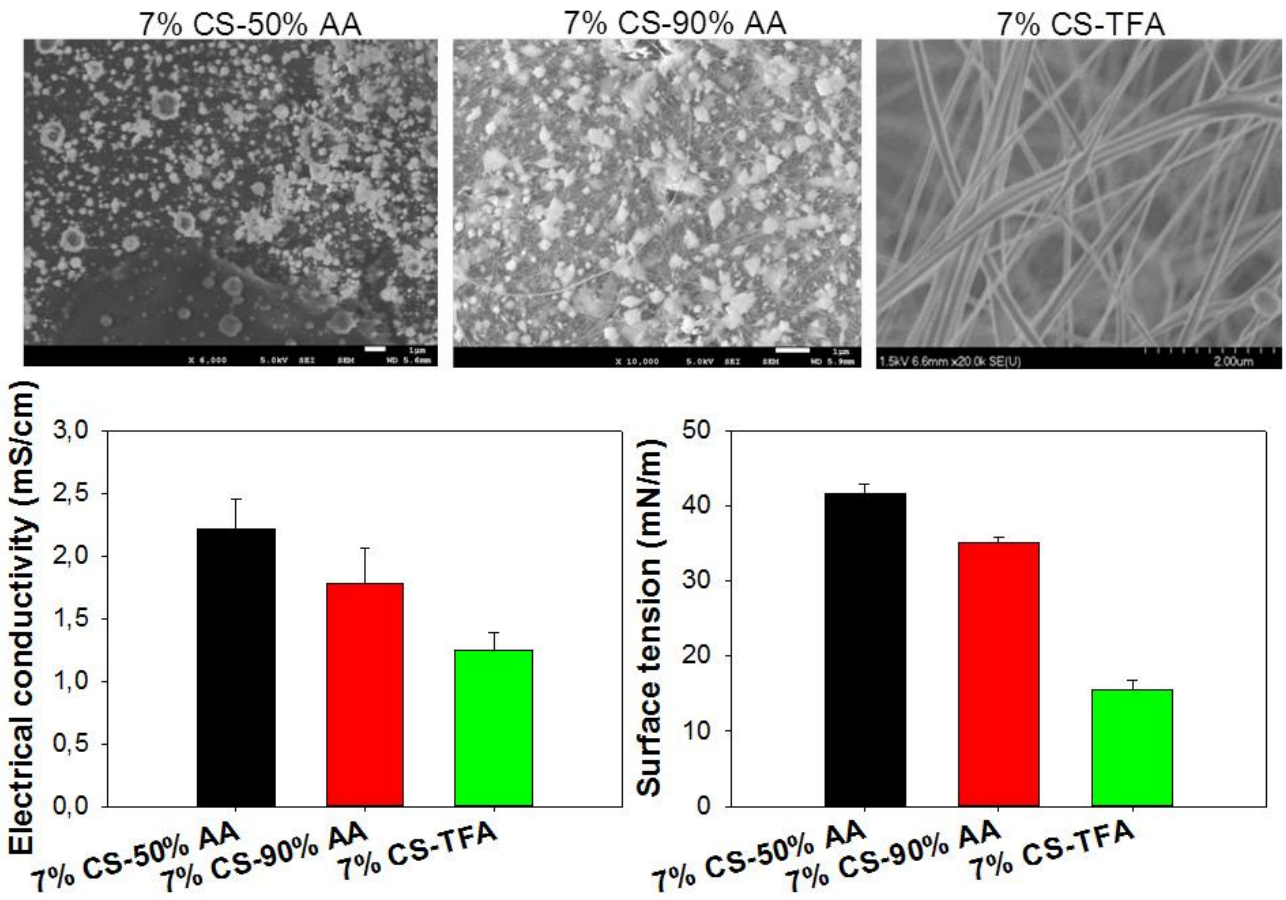

Figure 6.9: Top: Electrospinnability of neat CS solutions in 50 % AcOH, 90 % AcOH and trifluoroacetic acid (TFA); Bottom: Electrical conductivity and surface tension analysis of the same polymer solutions.

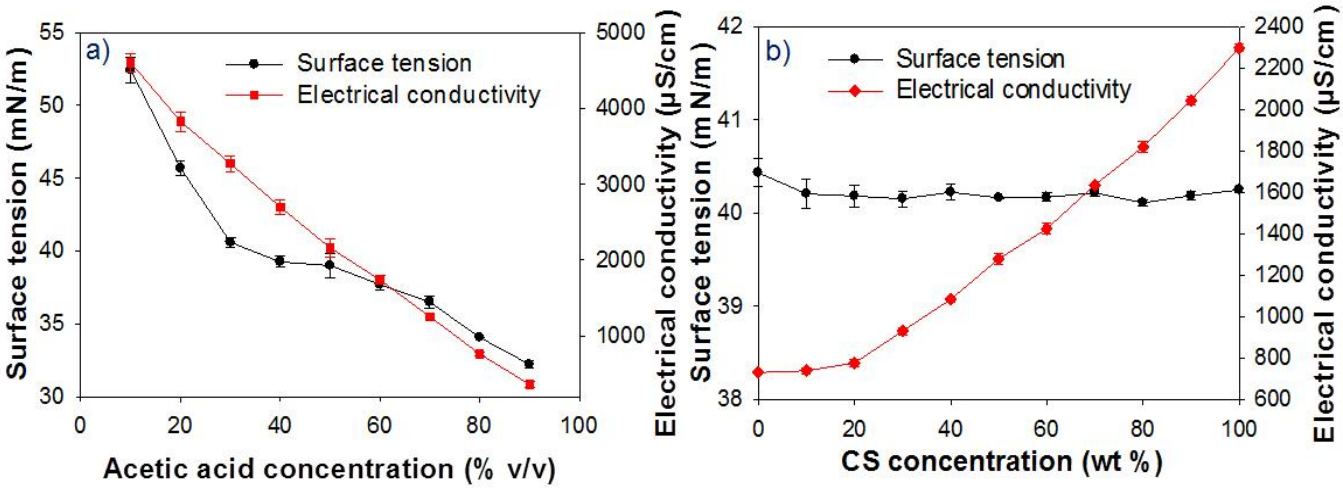

Figure 6.10: Electrical conductivity and surface tension of: a) CS solutions and b) CS/PEO blends as a function of the AcOH % v/v content and CS wt % in the CS/PEO blends, respectively.

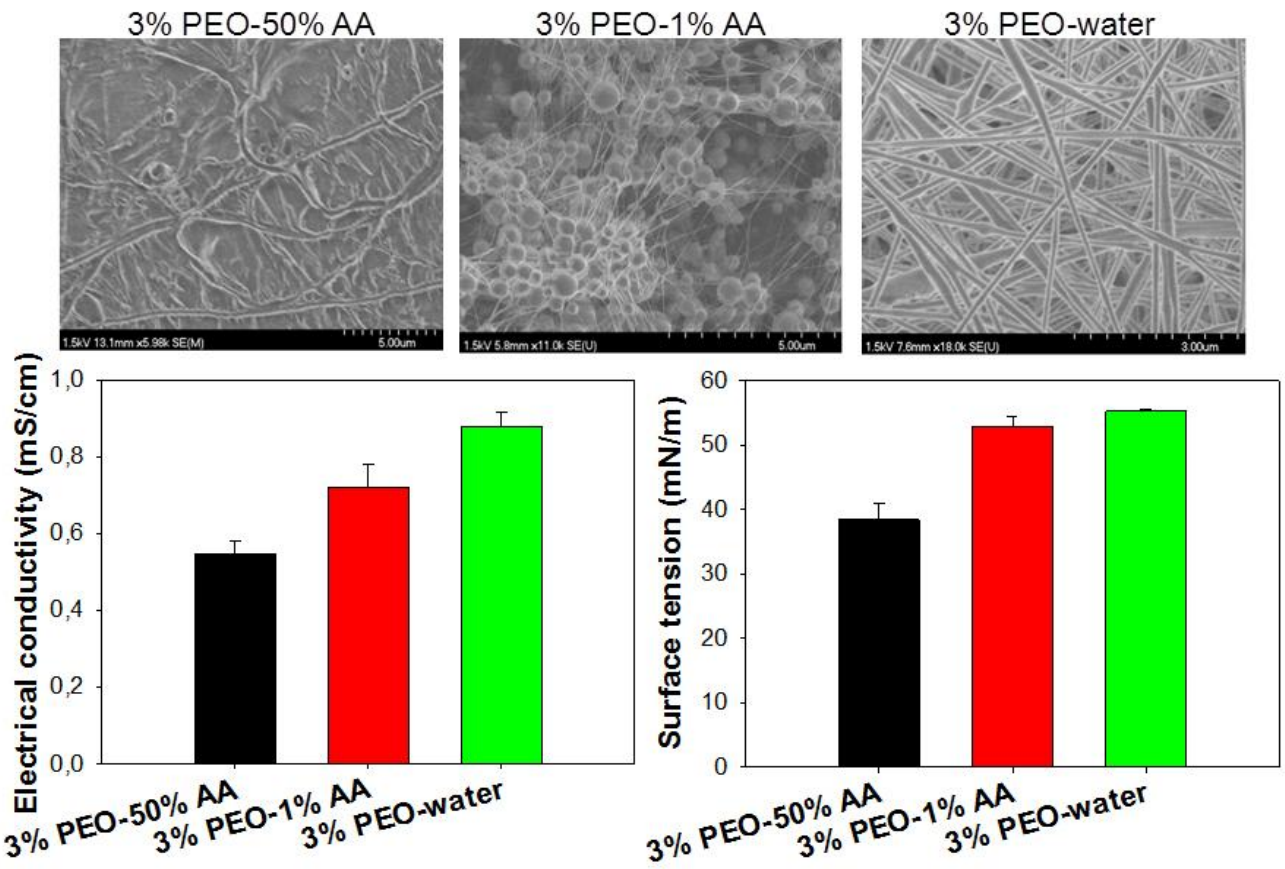

Figure 6.11: Top: Electrospinnability of 3 % (w/v) neat PEO solutions in 50 % AcOH, 1 % AcOH and water; Bottom: Electrical conductivity and surface tension analysis of the same polymer solutions.

CHAPITRE 7 DISCUSSION GÉNÉRALE

L'électrofilage du chitosane en vue de l'obtention de nanofibres est un procédé simple, facile et peu coûteux. Les nanofibres obtenues possèdent des propriétés remarquablement utiles et très recherchées pour diverses applications dans divers domaines. Malgré la simplicité du procédé d'électrofilage, l'obtention de nanofibres à base de chitosane (CNFs) est une tâche complexe et fastidieuse et ce en raison des multiples paramètres (conditions opératoires et paramètres intrinsèques des solutions) affectant le procédé. S'il est vrai que plusieurs études ont investigué l'effet des conditions opératoires sur la morphologie des fibres, à savoir le voltage, le débit, la distance aiguille-collecteur, la température, le degré d'humidité, ou encore l'influence de certaines propriétés physicochimiques et rhéologiques intrinsèques des solutions sur l'électrofilabilité du chitosane, notamment la concentration en polymère et en solvant, le DDA et le poids moléculaire du chitosane utilisé, la conductivité électrique des solutions d'électrofilage, leur tension de surface et leur viscosité, aucune ne rapporte l'effet de la conformation des chaînes de chitosane en lien direct avec leur protonation, densité de charge, flexibilité, enchevêtrement et élasticité sur la formation des nanofibres. Dans ce projet, des CNFs ont été préparées avec succès. L'évolution de la structure conformationnelle en fonction de la teneur en acide et de l'ajout de PEO a été étudiée en matière de densité de charge (quantifiée par le potentiel ζ) et d'élasticité (rapportée par tan delta) des chaînes polymères en solution.

Une fois le procédé optimisé, les meilleures formulations en termes d'électrofilabilité et d'activité bactéricide ont été sélectionnées pour la suite des tests. Dans cette étude, les propriétés antibactériennes et le mécanisme d'action des CNFs contre les bactéries Gram négatif et Gram positif les plus fréquentes dans l'industrie agroalimentaire ont été investiguées pour la première fois. La susceptibilité/résistance aux CNFs a également été examinée. D'autres éléments clés et questions jusque là sans réponses comme l'effet bactéricide ou bactériostatique du chitosane, l'influence de l'hydrophobicité des souches bactériennes utilisées, leur densité de charge négative de surface, mais encore leur pathogénicité et virulence ont été également prises en compte.

Nos résultats *in vitro* démontrent que le mécanisme d'action prédominant des CNFs est attribué à leurs groupements fonctionnels amines protonés, et ce indépendamment du type bactérien (Gram négatif ou Gram positif). Cependant, il n'est pas exclu qu'une possible solubilisation des CNFs contribue, à moindre effet, à leur action antibactérienne. Les chaînes de chitosane en solution

emprunteraient alors le même mode d'action que les nanofibres et agiraient sur la paroi bactérienne via leurs groupements amine. Il est également probable, mais toujours à moindre effet, que ces chaînes agissent *via* le mode d'action selon lequel le chitosane entoure et emprisonne la bactérie dans une enveloppe polymère l'empêchant de réaliser les échanges cellulaires nécessaires à sa croissance. Nos résultats indiquent fortement que la sensibilité des bactéries étudiées n'est pas Gram-dépendante, comme indiqué dans la littérature, mais plutôt souche-dépendante. En outre, par opposition à ce qui est disponible dans la littérature, nos résultats montrent que le chitosane possède un effet antibactérien irréversible et donc bactéricide et non bactériostatique. Les CNFs causent notamment la perméabilisation et la perforation de la membrane plasmique. En effet, la détection de matériel génétique (ADN), d'enzymes et de protéines intracellulaires dans le milieu externe est une indication de la perméabilisation de la membrane causée par les CNFs. De plus, la formation de pores au niveau de la paroi étant confirmée par les observations en microscopie électronique à transmission (MET), la perforation de la membrane a irrémédiablement conduit à la mort cellulaire.

Les CNFs étudiées ici se sont avérées très efficaces pour inhiber et empêcher la croissance des microorganismes sélectionnés à pH 5.8 (pH < p*K*a du chitosane). En revanche, cette efficacité s'est avérée strictement dépendante du pH (faiblement acide), ce qui représente un des inconvénients majeurs du CS et des CNFs. Pour parer à cette dépendance envers le pH, le chitosane quaternisé pouvant assurer la protonation permanente des sites cationiques indépendamment du pH du milieu serait une bonne alternative. Autrement, il est tout à fait envisageable et raisonnable de limiter l'utilisation des CNFs à des aliments ayant un pH intrinsèque faiblement acide tels que le lait, les jus de fruits, les yogourts, les fromages, les viandes et poissons, dont le pH s'acidifie à mesure que de l'acide lactique est libéré durant la conservation.

D'autre part, l'utilisation des CNFs dans le domaine biomédical a fait l'objet d'un projet CRSNG-Engage en 2016. À l'issue de ce projet, il a été montré, avec succès que les CNFs pouvaient être utilisées comme pansements antibactériens [11]. Le caractère non toxique de ce type de matériaux (CNFs) les rend particulièrement attrayants pour la filtration de l'eau ou encore l'industrie alimentaire, étant donné que l'interaction délibérée entre l'aliment et l'emballage est la définition même des emballages bioactifs.

Les tests antibactériens *in situ* ont montré le potentiel des CNFs en tant que nanomatériaux bioactifs barrière à la contamination de la viande et leur capacité à maintenir la qualité et à prolonger la durée de conservation de la viande rouge fraîche au-delà d'une semaine. Cependant, un élément pouvant limiter l'utilisation des CNFs comme emballage alimentaire actif est le fait que leur efficacité soit strictement conditionnée par un contact avec les aliments emballés. Ceci réduirait davantage les applications potentielles à certains types d'emballages comme l'emballage sous vide de produits tels que la viande fraîche, les saucisses, les charcuteries, les brochettes de poulet, les côtes, les viandes et saumon fumé, le poisson, etc. Pour surmonter ce frein, il est envisageable de combiner l'action antibactérienne des CNFs avec celle de certaines huiles essentielles pour un effet synergique.

L'autre écueil pouvant techniquement faire obstacle à l'application des CNFs comme partie intégrante d'un film multicouche est leur faible adhésion à la monocouche interne de l'emballage. Celle-ci est généralement constituée par du PE extrudé et la différence de polarité et d'hydrophobicité/hydrophilicité ajoutées à la mauvaise affinité entre les deux matériaux (CNFs et PE) sont à l'origine de leur incompatibilité. Un traitement corona ou plasma serait alors une issue qui s'impose afin d'y remédier.

En dépit de sa disponibilité et de ses nombreuses propriétés particulièrement attractives, le chitosane ne connait pas le même succès commercial fulgurant que l'amidon, la cellulose, l'alginate et le xanthane. En effet, ces polysaccharides sont disponibles et même très utilisés en tant qu'additifs alimentaires, comme épaississant et/ou stabilisant, ce qui n'est pas encore le cas pour le chitosane, bien que de nombreuses études aient réussi à démontrer les bénéfices pour diverses applications dans le domaine agroalimentaire. Les raisons possibles de la difficulté des produits à bases de chitosane à percer le marché sont discutées plus en détail dans ce qui suit.

La faible solubilité du chitosane dans des conditions de pH neutres et basiques peut limiter les applications de la plupart des systèmes à base de chitosane. Bien que les dérivés du chitosane, incluant le chitosane quaternisé, puissent représenter une solution alternative, leurs méthodes de production sont encore à l'étude et loin d'être commercialisées à des prix abordables à l'échelle industrielle. De plus, les réactions d'alkylation (quaternisation) et de carboxylation du chitosane font appel à des réactifs nocifs pour la santé et, qui plus est sont interdits par la FDA. Parmi ces

composés chimiques, on peut citer le borohydrure de sodium (NaBH4), *N*-méthyl pyrrolidone (NMP) et le glutaraldéhyde.

Par ailleurs, les faibles propriétés mécaniques, la perméabilité aux gaz et à la vapeur d'eau médiocres du chitosane peuvent limiter son utilisation sous forme de films pour des applications dans l'emballage alimentaire. Des recherches futures devraient être menées pour améliorer ces propriétés. En outre, la standardisation des grades de chitosane sur la source de chitine, le processus d'extraction, la saturation des sites fonctionnels, la disponibilité de la matière première au niveau industriel, les interactions possibles avec les aliments, sont des points critiques devant être considérés avant de prétendre à la commercialisation de matériaux à base de chitosane, du moins dans le cas des applications dans l'emballage alimentaire. En outre, les questions de réglementation en cours peuvent constituer un obstacle à l'utilisation du chitosane dans des applications aussi sensibles que l'industrie alimentaire. Le chitosane n'étant pas encore approuvé par Santé Canada.

Justement, d'un point de vue réglementaire, comme le chitosane est commercialement disponible en tant que produit amincissant et aidant à maintenir des niveaux de cholestérol sains, Santé Canada recommande toutefois une posologie spécifique. Il est donc préférable de le prendre pendant les repas, et en cas de médication ou de prise de suppléments ou autres produits de santé naturels, de le prendre deux heures avant ou après, puisqu'il peut retarder ou empêcher l'absorption de certains médicaments, vitamines ou oligoéléments, raison pour laquelle il est déconseillé chez la femme enceinte [127].

Un dernier détail mais non des moindres pouvant expliquer le manque de produits à base de chitosane sur le marché Nord Américain actuel est sans doute le prix du biopolymère. En effet, le prix moyen actuel du chitosane s'élève à 25 $/lb. À titre comparatif, un film à base de polyéthylène (PE) synthétique tourne autour de 2.50 $/lb. Le coût du chitosane revient alors dix fois plus cher qu'un PE couramment utilisé dans le domaine de l'emballage. Cette situation est comparable au cas du polylactide (PLA) au tout début de son lancement car en effet, le PLA avait du mal à concurrencer le marché du polyéthylène téréphtalate (PET) bien plus abordable et disponible. En revanche, suite à de nombreuses entrevues réalisées dans le cadre d'une étude de marché, réalisée en parallèle à ce projet, aussi bien avec des transformateurs alimentaires (viande, produits laitiers, plats préparés et salades fraîches, etc.), qu'avec des fabricants d'emballages ou

encore des experts/consultants dans le domaine, il en ressort que la demande pour des emballages plus écologiques (naturels, biosourcés, biodégradables, etc.) pouvant prolonger la durée de conservation des aliments est aujourd'hui plus forte que jamais. On devrait donc s'attendre à une croissance du marché du chitosane dans les années à venir (tel que mentionné dans la littérature).

Enfin, dans l'ensemble, prolonger la date limite de conservation (DLC ou « meilleur avant ») grâce à ce type d'emballage pourrait aider au développement d'aliments naturels, peu ou pas transformés, sans additifs ni conservateurs et satisfaire ainsi la demande croissante des consommateurs pour ce type de produits. Ceci faciliterait grandement la logistique derrière toute la chaîne de production, y compris la distribution et le stockage. En plus de réduire le gaspillage et les pertes financières qui y sont associées, ce gain dans la durée de vie des aliments permettrait aux producteurs et transformateurs alimentaires d'augmenter leur chiffre d'affaires en exportant sur de plus longues distances, tout en améliorant la qualité et la salubrité du produit emballé pour la santé des consommateurs. Ainsi, l'étude de marché (plus de 30 compagnies interrogées) a permis de valider le besoin criant de l'industrie alimentaire vis-à-vis des avantages qu'offre ce type d'emballage. En effet, sachant pertinemment que les consommateurs achètent des aliments dont la durée de vie est la plus longue, il n'est d'ailleurs pas surprenant que les producteurs et transformateurs alimentaires veuillent utiliser cet avantage comme un argument de vente concurrentiel leur permettant de dominer le marché.

CHAPITRE 8 CONCLUSIONS, PERSPECTIVES ET RECOMMANDATIONS

8.1 Conclusions et perspectives

Les résultats de cette étude montrent que l'activité antibactérienne des nanofibres de chitosane (CNFs) est attribuée à leurs groupes fonctionnels NH_3^+ et à la rupture et perforation de la membrane bactérienne. En conséquence, cela entraîne la fuite des composants intracellulaires tels que protéines et nucléotides. La biodisponibilité des fonctions NH_3^+ des CNFs favorise et maximise leur adhésion à la surface cellulaire (de charge opposée) par le biais des attractions électrostatiques. Le modèle établi dans cette étude en ce qui concerne le mode d'action des CNFs, suggère que les bactéries, mues par les interactions électrostatiques, migrent vers la surface des CNFs pour les utiliser comme support pour leur croissance et multiplication. Celles-ci se retrouvent alors piégées dans le réseau tridimensionnel des CNFs, en raison de leur grande surface spécifique et porosité élevée.

Nos résultats suggèrent également que l'adsorption du chitosane à la surface de la paroi bactérienne est la première étape dans le mécanisme d'action des CNFs, suivie par la perforation de la membrane, la fuite des composés cytosoliques et ultimement la lyse et la mort cellulaire. Néanmoins, il n'est pas exclu qu'une partie de l'activité antibactérienne puisse être due à une dissolution partielle des nanofibres qui rendrait le chitosane disponible en solution.

Les CNFs représentent des biomatériaux prometteurs pouvant réduire les infections microbiennes d'origine alimentaire ainsi que l’altération microbiologique des aliments, deux enjeux de santé publiques majeurs, souvent à l’origine du gaspillage alimentaire. Un gaspillage auquel toutes les sociétés, en particulier nos sociétés occidentales font face actuellement, un gaspillage auquel l’ONU a pris les engagements nécessaires afin d’y remédier, ou du moins le réduire. À titre d’exemple, l’industrie de la viande fait présentement des pertes qui s’élèvent à entre 5 et 25 % de leur production totale, juste parce que la DLC est expirée ou que l’aspect visuel de la viande n’est plus bon ou encore en raison d’une contamination microbienne. Des pertes qui se chiffrent en plusieurs millions de dollars et la situation est semblable dans les autres secteurs (viandes biologiques, lait, fromages, poissons, poulet, etc.).

Les CNFs développées durant ce projet permettent de prolonger la DLC de la viande fraîche de plus d'une semaine, sans pour autant altérer la qualité organoleptique de l'aliment. Ce gain non négligeable permettrait de diminuer le recours excessif aux agents de conservation utilisés dans l'industrie de la viande dont le lactate et diacétate de sodium, et qui plus est ne sont pas nécessairement étiquetés sur la liste des ingrédients ou encore les nitrates et les nitrites qui, on le sait maintenant sont nocifs pour la santé. Leur caractère non toxique rend les CNFs particulièrement attrayantes pour l'emballage alimentaire, étant donné que l'interaction délibérée avec l'emballage est la condition voire la définition-même des emballages actifs.

Le prochain défi consiste à produire les CNFs à grande échelle en utilisant un dispositif d'électrofilage industriel pour des tests de faisabilité, de rendement et de performance. Un prototype multicouche à base de CNFs combinées à d'autres polymères afin de parer aux faibles propriétés mécaniques et barrière du chitosane pourrait être conçu et testé avant la validation finale contre les bactéries incriminées dans les toxi-infections alimentaires et l'altération microbiologique des aliments dont le poulet, les fruits et légumes, les aliments transformés, le lait, les jus de fruits, etc.

8.2 Recommandations

Les points suivants sont *recommandés* pour les travaux futurs :

1) Étudier l'internalisation du chitosane à travers la paroi vers la membrane. Déterminer le rôle du lipopolysaccharide (LPS) ainsi que les protéines et les voies métaboliques impliquées.

2) Tester l'activité AB des CNFs *in situ* sur d'autres types d'aliments (fruits, légumes, poulet, poisson, etc.) et sur d'autres bactéries dont *S.* Typhimurium, *L. innocua*, *S. aureus*, *P. aeruginosa*, *P. fluorescens* et notamment des souches provenant de la banque American Type Culture Collection (ATCC).

3) Suivre l'évolution de la flore totale et lactique intrinsèques de la viande en présence et en l'absence de CNFs pour évaluer le degré d'altération du produit ainsi que l'efficacité bactéricide des CNFs et l'allongement de la DLC de la viande. Dans la même optique, il serait également pertinent de suivre l'évolution de la flore psychrotrophe, étant donné que les tests ont été réalisés à la température de réfrigération, à savoir 4 °C.

4) Tester l'efficacité AB clinique (sur des patients malades ou des animaux) des CNFs en tant que pansements antibactériens.

5) Investiguer les propriétés rhéologie élongationnelle des solutions d'électrofilage, étant donné que durant le procédé, la déformation encourue est plus de l'élongation que du cisaillement.

6) Améliorer la compatibilité du chitosane avec le PE, PP, etc. et ce en faisant un traitement de surface type corona en vue d'améliorer l'adhésion des CNFs à la surface des emballages conventionnels.

7) Évaluer les propriétés d'absorption d'eau des CNFs au contact de l'aliment pour apprécier les propriétés hémostatiques et de gonflement des nanofibres (par microscopie à force atomique ou AFM).

8) Évaluer l'effet des CNFs sur les propriétés organoleptiques des aliments testés en termes d'odeur, saveur, aspect visuel et texture.

9) Évaluer l'effet du chitosane sur les propriétés nutritionnelles des CNFs et ce en se penchant sur les interactions chitosane - lipides/protéines/minéraux car en effet, le pouvoir chélatant du CS pourrait diminuer la biodisponibilité de ces nutriments voire même causer la désorption de leur contenu dans l'organisme et ainsi provoquer des carences. À cet effet, il serait pertinent d'utiliser le simulateur du tube digestif du Pr. Ismail Fliss.

10) Déterminer le degré de saturation des sites fonctionnels des CNFs aussi bien *in vitro* que dans les conditions réelles (en contact avec les aliments). La difficulté dans cette recommandation réside dans le fait qu'il serait difficile de différencier les unités glucosamines du CS de celles des aliments (protéines, peptides et acides aminés), à moins de marquer ces unités et suivre leur saturation par des techniques comme la fluorescence après photoblanchiment (ou Fluorescence Recovery After Photobleaching, FRAP) ou encore le transfert d'énergie entre molécules fluorescentes (Fluorescence Resonance Energy Transfer, FRET).

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